FINAL REPORT

Washington State Grape and Wine Research Program

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Email to: ARCGrants@wsu.edu

Project:

Project Title: Microbiology and Chemistry of Washington Wines

Project Duration: FY 2015-2018

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PI Name:	C.G. Edwards
Organization	Washington State University
Address	School of Food Science
Telephone	509-335-6612
Email	edwardsc@wsu.edu

Cooperator Name:	B. Bondada	**Cooperator Name:**	C.F. Ross
Organization	Washington State University	Organization	Washington State University
Description of participation:	Student committee member	Description of participation:	Sensory methods development/student committee member

Cooperator Name:	G. Unlu	**Cooperator Name:**	
Organization	University of Idaho	Organization	
Description of participation:	Microbial physiology/ student committee member	Description of participation:	

Final Financial Reporting (Budget):

	Year 1 FY 2015-16	**Year 2 FY 2016-17**	**Year 3 FY 2017-18**
	July 1 – Jun 30	July 1 – Jun 30	July 1 – Jun 30
Total	$50,000	$50,000	$59,754
Footnotes:			

Total Project Funding: $159,754

Project Budget Status: Available funding is currently on track to be consumed by approximately July 2018.

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Project Summary:

The yeast, *Brettanomyces bruxellensis*, spoilage is a concern, if not a major threat, to red wine quality. Additional research regarding those factors that impact its growth and ability to survive under various conditions would allow for prediction of high-risk situations as well as development of effective control measures. Thus, this project evaluated the ability of this yeast to survive in winery waste (*e.g.*, pomace) sometimes spread in vineyards over a lengthy storage time (>1 year).

A major vector for *B. bruxellensis* has been oak barrels. Given the porous nature of wood, the yeast can penetrate staves such that the effectiveness of methods for cleaning and sanitizing are questionable. During this project, new barrels were infected with strains of *B. bruxellensis* originally obtained from Washington wines. After a lengthy storage with wine, the barrels were drained and taken apart to determine the penetration depth of the yeast as well as impacts of such sanitizing protocols as steaming or hot water on recovery of viable cells. Results from this project have yielded suggested times/temperatures for removing the yeast from oak barrels.

Worldwide, some winemakers are purposefully encouraging the growth of non-*Saccharomyces* yeasts present on grapes to improve such sensory parameters as 'mouthfeel.' More recently, researchers in Australia have reported success in reducing alcohol concentrations by using sequential fermentations, *i.e.*, inoculation with selected non-*Saccharomyces* species followed by addition of *Saccharomyces*. While winemakers can rely on native yeasts to provide unique and desirable sensory qualities, there is a risk to quality if undesirable non-*Saccharomyces* yeasts dominate fermentation. In addition, non-*Saccharomyces* are being investigated to lower final ethanol concentrations wines. Here, many of these yeasts will utilize fermentable sugars without production of ethanol thereby lessening the amount of sugar *Saccharomyces* needs to ferment to achieve dryness.

Project Major Accomplishments:

<u>Objectives</u>

1. *Brettanomyces* spoilage of wines.

    (a) Continue to examine whether new technology (so-called "electronic tongue") can be used for detection of *Brettanomyces* spoilage.

    (b) Evaluate seasonal survivability of the yeast over time in grape pomace samples located in Washington State vineyards.

    (c) Assess the effectiveness of physical treatments to eliminate the yeast at different depths in oak barrel staves that differ by oak type and toasting level.

2. Impact of non-*Saccharomyces* yeasts on wine quality.

    (a) Ability of sequential inoculations of non-*Saccharomyces* and *Saccharomyces* as a means to lower final alcohol yield.

(b) Evaluate nitrogen and sugar utilization of sequential inoculations of non-*Saccharomyces* and *Saccharomyces* yeast during aerated and non-aerated growth.

(c) Determine impact of non-*Saccharomyces* yeast on mouthfeel due to increased solubility of pectins.

Objective 1

Objective 1a: In agreement with prior industry comments, experiments in support of this objective were not considered a priority. However, some experimentation was conducted with C.F. Ross related to the ability of the electronic tongue to detect *Brettanomyces* using wines produced as part of Objective 1c. Here, discriminant function analysis found significant differences between the wines based on toasting level and oak type, with more variability coming from American than French oaks (Figure 1). When comparing barrels of same oak source (American or French) and toasting level (none or medium), differences in responses were also noted. Compared to wines inoculated with strain I1a, samples with strain E1 showed higher responses from the bitter, sweet, spicy, salty and umami sensors although concentrations of volatile phenols were similar between these wines (data not shown).

Objective 1b: Experiments regarding the survivability of *B. bruxellensis* in grape pomace initiated in 2014 were concluded in 2017. Briefly, grapes (cultivar Syrah) were obtained from a research vineyard located at IAREC (Prosser, WA) and crushed/destemmed using standard methods. *S. cerevisiae* strain D254 was inoculated at 10^6 cfu/mL with pomace collected from pressings at two different times; early (11.5% v/v alcohol) or late (14.9% v/v alcohol). From each pressing, pomace samples (100 g) were transferred into sterilized, plastic containers fitted with filtration membranes that allowed the exchange of water/gases but not microorganisms. Half of these containers was sterilized by high dose gamma irradiation (25 kGy). Both non-sterilized and sterilized pomace samples were inoculated with 5×10^4 CFU/g *B. bruxellensis* I1a and incubated at room temperature for one week before being placed into two vineyards located in the Columbia Valley AVA in November, 2014. From that time, representative containers were removed in triplicate during 2015 (January, April, June, August, and November) and 2016 (January, March, May, August, October). Additional samples were placed into a third vineyard in the Walla Walla Valley AVA in November 2015 based on the same protocol. Once the vineyard samples were retrieved, pomace temperatures were equilibrated to 22° to 25°C for 24 hr before addition of 50 mL of a recovery medium (Renouf and Lonvaud-Funel, 2007). After an additional incubation of 24 hr, culturability was determined by standard plating methods using WL-D medium (Difco, Detroit, MI).

Environmental temperatures generally correlated with yeast populations during the first year with increases during the spring and summer months (Figure 2A). Soon after peak populations were reached (Figure 2B and 2C), recovery of *B. bruxellensis* steadily declined regardless of vineyard temperatures, possibly due to a lack of required nutrients (*e.g.*, biotin, nitrogen-containing compounds, etc.) or build-up of toxic metabolic by-products. *B. bruxellensis* were not initially recovered from samples collected when average vineyard temperatures were below freezing. However, additional incubation of these pomace samples in recovery medium led to growth of the yeast, indicating that populations were initially quite low but still viable.

As small, viable populations of *B. bruxellensis* inoculated into grape pomace could be recovered, these findings further support the contention that the yeast can survive in vineyards, even under harsh conditions. Whether the yeast can freely move from pomace to grapes prior in subsequent harvests has not been proven. However, this would be a reasonable assumption given that the yeast can move with air currents (Connell et al. 2002).

Heating pomace as a means to remove the yeast did, in fact, reduce recovery of *B. bruxellensis* depending on the temperature, length of heating and the addition of SO_2 (Figures 3 to 6). At 40°C (Figure 3), populations declined by 1 to 2 logs after heating for 1 to 5 min. Although culturable populations further decreased with 45 mg/L total SO_2 added, *B. bruxellensis* could be recovered from all heat treatments up to 10 min. At 50°C (Figure 4) and 60°C (Figure 5), heating for a minimum of 5 min greatly reduced culturable populations while the presence of sulfite consistently reduced culturability in comparison to pomace without added SO_2. The yeast was eliminated from pomace heated for 1 min at 70°C (Figure 6) as evidenced by no recoverable cells even after 60 days of incubation in EBB medium. In contrast to heating of pomace to 50° or 60°C, the presence of sulfites did not have a noticeable effect as culturable cells were not recovered regardless of total SO_2 concentration.

Unlike temperature and the presence of sulfites, only slight differences in cell recoveries were noted between pomaces containing different amounts of alcohol (*i.e.*, 11.7% or 15.1% v/v). Generally, pomace coming from higher alcohol wines had smaller yeast populations compared to pomace from lower alcohol wines at equivalent levels of total SO_2. Hence, a treatment of 40°C for 10 min to pomace containing 11.7% or 15.1% v/v alcohol resulted in initial recovered populations of approximately 10^2 or <30 cfu/mL, respectively (Figure 5). Similar examples where populations below detection limits correlated with higher alcohol pomace were observed at other temperatures, especially at 50°C after 1 or 5 min of heating (Figure 6). Among all the heating trails, there was only one treatment where higher alcohol concentration completely changed heating requirements to remove *B. bruxellensis* from pomace. In fact, sulfited samples held at 40°C for 30 min still had culturable yeast in pomace containing 11.7% v/v alcohol but not 15.1% v/v (data not included).

This research further emphasizes the need to microbiologically treat pomace to destroy residual viable cells if the material (pomace, lees, etc.) is to be returned to vineyards.

Objective 1c: Four 225-L barrels (duplicate barrels of French medium-low toast and American medium-high toast) infected with *B. bruxellensis* were obtained from a regional winery. While populations present in these staves were low, *B. bruxellensis* was recovered from 0 to 4 mm layers obtained from top (Figure 7A) or bottom (Figure 7B) staves. As previously observed with the 16-L barrels, yeast populations entered logarithmic growth quicker in wines incubated with pieces of French oak staves compared to American oak. Unlike prior results using 16-L barrels, the yeast was recovered from the commercial barrels at a greater depth of 5 to 9 mm.

Additional analyses of these staves were conducted using a Forstner drill bit which leaves behind a flat surface. Shavings from drilling 1-inch diameter holes were carefully collected at various depths and placed in EBB recovery media. Two different methods to recover yeasts from oak shavings were tested; (a) simple mixing with sterile wine for 12 hours and (b) stomacher mixing

with recovery media for 3 minutes. No statistical differences were noted between these two methods.

In general, yeast populations and penetration depended on the barrel type (French vs. American) and size (16-L vs. 225-L) as illustrated in Table 1. For 16-L barrels, while French heavy toasted oak held a population of 1.9×10^3 cfu/mm^3 within the first 0 to 8 mm layer, the American light toasted was comparable, 1.2×10^3 cfu/mm^3. Populations were greatest in the 0 to 2 mm layers while lowest at 4 to 8 mm depths and was not recovered beyond 8 mm. In contrast, lower populations of *B. bruxellensis* were recovered from the commercial 225-L barrels (Table 1). Here, the French medium-low toasted oak had higher populations (204 cfu/mm^3) than the American medium-heavy toasted (55 cfu/mm^3) recovered from 0 to 8 mm layers. Unlike the 16-L staves, larger populations were found in the 2 to 4 mm and 4 to 6 layers compared to the 0 to 2 mm layer, possibly due to sanitation procedures used at the winery which may have lowered surface populations (ozone followed by SO_2 gassing).

Observations were made regarding the penetration of wine pigments as well as yeast morphologies within the staves themselves (Table 1). Penetration of wine into staves as noted by pigment migration was lowest for the American low toasted oak (3.5 mm) and highest for French medium low toasted (5.2 mm). However, pigment penetration did not necessarily correlate with recovered populations. For example, yeasts penetrated further into the staves than pigments for the 16-L French low toast and 225-L French medium-low toast staves. Stave pieces were also examined using scanning electron microscopy (SEM) to investigate structural differences between staves which could account for yeast population differences. Longitudinal cross sections of American low toasted (Figure 8A) and French heavy toasted (Figure 8B) show increased crevasses and cracks in the French oak may allow further penetration of *B. bruxellensis*. When focused on xylem vessels (Figure 8C and 8D), large numbers of yeast cells occupied the pore-like structures with some branched structures were observed (Figure 8E). The presence of pseudohyphae (branched structures) could allow deep penetration into porous oak by the yeast. The greatest depth where suspected *B. bruxellensis* was observed between 6 to 8 mm (Figure 8F). In conclusion, the yeast can penetrate oak staves up to approximately 8 mm depending on age (use) of barrel as well as oak type (French vs. American) and toasting level.

One means to eradicate the yeast from oak barrels is the use of heat applied in the form of (a) steam, (b) hot water, or (c) warm wine. To conduct these experiments, a circular stainless steel plate ($^3/_{16}$ x 35 inches) was designed and built with 16 rectangular slots which fit individual 3 x 10 cm stave blocks. Once placed over a steam kettle containing boiling distilled water, the plate was loaded and steamed for 0, 3, 6, 9 or 12 min (30 cm between water level and bottom of blocks) in support of treatment (a). Specialized thermocouples were placed into horizontally drilled holes at various depths (0 mm or inner surface, 4.5 mm, 9.5 mm, 14.5 mm, and outer surface). After steam treatment, blocks were sawn into 4 mm layers and individually placed in a nutrient-rich, sterile red wine. Yeast culturability in this recovery wine was evaluated for up to 60 days. For treatments (b) and (c), the steel plate was loaded and placed into 4 L of circulating hot distilled water (50°, 60°, 70° or 80°C) or warm Cabernet Sauvignon (35°, 40°, 45° or 50°C) for up to 120 min. During heating, samples were randomly removed in triplicate every 5 min (hot water) or 15 min (warm wine). Experiments at each temperature were conducted three separate times. Furthermore, the ethanol concentration of the warm wine was varied (11% or 15% v/v).

Thermocouple data during steaming illustrated the lag times in heating between the inner and outer surface of staves. Using steam, little variation was noted between staves from 16-L barrels and 225-L barrels, either American medium-high toast (Figure 9A) or French medium-low toast (Figure 9B). On average, three to four minutes was required for layers <9 mm to reach at least 55°C, a temperature which *B. bruxellensis* is thought to have a D-value (time for 90% reduction in population) of approximately 1 min. Heat penetration using water (Figure 10) or red wine (Figure 11) depended on the temperature utilized. While the inner most layer (0 to 4.5 mm) quickly approached the temperature of the heated liquid, outer layers (14.5 to 25 mm) were generally unaffected. For example, ~20 or 30 minutes were required to reach 55°C for layers ≤9.5 mm using 70° and 80°C hot water, while this temperature was never reached at 9.5 mm depths for warm wine treatments after 120 min. Like with steam, differences in rate of heating were not observed for staves of different oak species, toasting levels, size, or age.

After heating, blocks were sawn into layers to recover any viable cells of *B. bruxellensis* remaining. As expected, the length of steam exposure greatly influenced recovery of *B. bruxellensis* from 16-L or 225-L barrel staves (Figures 12 to 14). For the 16-L staves, as steaming times increased, the number of days in the nutrient-rich wine required to detect the yeast also increased. For instance, steaming staves for <6 min yielded in maximum populations of approximately 10^6 cfu/mL after incubation of 60 days in the nutrient-rich wine. Additional steaming staves to 6 to 9 min resulted in no recovery of *B. bruxellensis* from the 0 to 4 mm layer from American oak (Figure 12). When present in the 5 to 9 mm layer in French oak (Figure 13), an additional 3 to 6 min of steaming beyond that required for the 0 to 4 mm layer was necessary to not recover any cells. Statistical differences between the two strains of *B. bruxellensis* present in 16-L staves, I1a or E1, were not apparent (data not included). Compared to the 16-L staves, longer incubation periods and lower populations were noted oak pieces from 225-L barrels (Figure 14) but similar trends were observed.

Correspondingly, the length of hot water or warm wine exposure determined *B. bruxellensis* recovery from infected barrel staves (Table 2). Like with steaming, recovery of viable yeast cells from staves layers was monitored for up to 60 days after treatment. If present in staves at depths of 0 to 4 mm, exposing staves to hot water at 50° (60 min), 60° (30 min), 70° (20 min) or 80°C (25 min) resulted in culturable cells not being recovered. However, longer exposure times were necessary at each temperature if the yeast was present 5 to 9 mm to no longer recover viable cells. However, use of 50°C water never completely eradicated *B. bruxellensis* even after 90 min. In comparison, no viable cells could be recovered from blocks treated with warm wine at 50°C for 60 min (wine A) or for 45 min (wine B), the latter having a higher amount of ethanol. In fact, eradication of the yeast from the 0 to 4 mm layer required shorter times when using wine with 15% v/v ethanol compared to that with 11%. If the yeast had penetrated deeper into the stave (*i.e.*, 5 to 9 mm), eradication only occurred in wine at 50°C for 120 minutes no matter the concentration of ethanol present.

Based on these results, a steaming time of at least 12 minutes was needed to remove *B. bruxellensis* if yeast are present in staves at <9 mm depths. Additional steaming time would be required if the yeast were present >9 mm. Alternatively, hot water and warm wine treatments may serve as hurdles towards *B. bruxellensis* oak infections if appropriate time and temperature combinations are used (*i.e.*, 70° or 80°C water for 30 or 20 min, respectively, or 50°C wine for 120 min).

Objective 2

Objectives 2a and 2b: Grape juice concentrate (Merlot, California Concentrate Company, Acampo, CA) was reconstituted and adjusted to 155 g/L glucose, 155 g/L fructose, pH 3.58 (tartaric acid), and 270 mg N/L YAN (150 mg/L amino N and 150 mg/L NH_4^+). The reconstituted juice was divided into five-gallon buckets and frozen at -20°C until needed. Upon thawing, the juice was sterile-filtered through 0.45 µm (Millipore, Billerica, MA) into 6L bioreactors containing 20 µm Sigmacell cellulose (1 g/L, Sigma-Aldrich, St. Louis, MO). Yeast starter cultures were prepared in liquid media and inoculated the juice at 10^4 to 10^5 cfu/mL. To half of the fermentations, oxygen was sparged at a rate of 0.025 L O_2/L juice/min. After six days at 21°C, 100 mL aliquots were transferred to sterile milk dilution bottles and inoculated with *Saccharomyces cerevisiae* strain (ICV D254, Lallemand, Montréal, Quebec, Canada). Residual amino acid nitrogen was quantified using Primary Amino Nitrogen kits (Megazyme International Ireland, Wicklow, Ireland) while ammonium was measured using an ion selective ammonia electrode. Concentrations of glucose, fructose, acetic acid, glycerol, and ethanol were measured by HPLC using an Agilent 1100 HPLC system (Agilent Technologies, Inc., Santa Clara, CA) equipped with quaternary pump, autosampler, column thermostat, refractive index detector, and ChemStation software and a BIO-RAD Aminex Fermentation Monitoring Column (Hall and Reuter, 2007).

Consumption of glucose and/or fructose by the non-*Saccharomyces* yeasts varied with higher amounts of glucose consumed compared to fructose (Tables 3 and 4). Lesser amounts of sugars were consumed under aerobic conditions compared to non-aerobic for some yeasts (*e.g., Ca. californica, Ca. railenensis, H. uvarum, I. orientalis, K. marxianus, Mt. chrysoperlae, P. fermentans, P. kluyveri, P. membranifaciens,* and *S. cerevisiae*) but in contrast to others (*e.g., Ca. oleophila, Cr. adeliensis, Cr. saitoi, Cu. pallidacorallinum, Mt. pulcherrima, My. carribica, My. guillermondii, W. anomalus*, or *Y. mexicana*). For *Cr. adeliensis, Cr. saitoi,* and *Cu. pallidacorallinum*, less carbohydrate utilization was expected without aeration as these yeasts are incapable of fermentation. Negligible ethanol production with or without aeration confirmed that sugar was not utilized for fermentative energy production. For other yeasts (*Ca. oleophila, My. guillermondii, My. carribica, W. anomalus,* and *Y. mexicana*), increased carbohydrate utilization with aeration suggests increased energy demand. In general, nitrogen utilization was higher under aerobic conditions while doubling time for most yeasts was not impacted by aeration condition with some exceptions (*Ca. californica, H. uvarum, K. marxianus, Mt. chrysoperlae, P. kluyveri,* and *P. membranifaciens*). Of importance were differences in ethanol production as expressed per g of sugar consumed. Here, some yeasts (*Ca. oleophila, I. orientalis, My. guillermondii, P. fermentans,* and *Y. mexicana*) produced far lower amounts of ethanol under aerated conditions while exhibiting little difference in doubling time. This indicated that more sugar was utilized for biomass rather than production of ethanol.

Under aerated conditions, several yeasts produced less ethanol compared to the control treatment (*i.e., S. cerevisiae* alone) while consuming similar amounts of sugar (Tables 5 and 6). However, aerated treatments generally yielded concentrations acetic acid well above the sensory threshold. For example, yeast combinations of *H. uvarum, K. marxianus,* and *W. anomalus* achieved both reduced ethanol and high sugar utilization with and without aeration but also elevated acetic acid levels (1.19 to 1.39 g/L). Other combinations utilizing *Cr. adeliensis, Cr. saitoi,* and *Cu. pallidacorallinum* yielded less ethanol (13.7 to 14.6% v/v) but also produced obvious bad

aromas. Based on ethanol reduction, acetic acid production, high sugar utilization, and absence of obvious foul odors, *Mt. chrysoperlae, Mt. pulcherrima, My. guillermondii, P. kluyveri,* and *P. membranifaciens* were chosen for additional screening.

For this screening, additional strains of *Mt. pulcherrima, Mt. fructicola,* and *Torulaspora delbrueckii* strain provided by Lallemand (Montréal, Canada). Grape juice concentrate (Merlot) was reconstituted and adjusted to 24.3°Brix with equal amounts of glucose:fructose and titratable acidity to 6.96 g/L using an 80:20 tartaric:malic acid mixture (final pH = 3.62). Sterile-filtered juice (300 mL) was dispensed into 500 mL media bottles containing 1 g/L Sigma-Cel. Starter cultures were prepared and used to inoculate juice at 10^6 cfu/mL in triplicate. Fermentation vessels were capped with a gas-porous Bugstopper® (Whatman, Maidstone, U.K.) to simulate open-top fermentation conditions and incubated at 20°C on a rotary shaker (100 rpm). After 72 hours, grape juices were inoculated with *S. cerevisiae* ICV D254 with stoppers replaced by fermentation locks and shaking discontinued.

Initial inoculation of non-*Saccharomyces* yeasts followed by *S. cerevisiae* significantly ($p \leq 0.05$) reduced ethanol content compared to those produced by *S. cerevisiae* alone (Table 7). Here, *Mt. pulcherrima* P01A016 achieved the greatest reduction, producing 11.7% v/v compared to 13.7% v/v by wines fermented only by *S. cerevisiae*. Ferments inoculated with *My. guillermondii* P40D002, *Mt. pulcherrima,* and *Mt. fructicola* reduced ethanol to yielded 12.1% to 12.27% v/v while *P. membranifaciens, P. kluyveri, Mt. chrysoperlae,* or *T. delbrueckii* strain reduced alcohol to 13.0 to 13.4% v/v. All yeast strains tested produced acetic acid at levels below the sensory threshold of 0.7 g/L, although higher levels were noted for *Mt. chrysoperlae, T. delbrueckii* and *S. cerevisiae*. Titratable acidities were found to increase without increases in acetic acid for wines with *Mt. pulcherrima* or *Mt. fructicola* (unknown reasons). Based on reduced ethanol and acetic acid production, native strains *Mt. pulcherrima* P01A016 and *My. guillermondii* P40D002, as well as the industrial *Mt. pulcherrima* strain, were selected for further winemaking trials 2016-2017.

Larger fermentations (200L) were conducted in 2016 and in 2017 to produce enough wine for both chemical and sensory analyses. *Mt. pulcherrima* A016 and *My. guillermondii* P40D002 originally isolated from a vineyard in Washington state while *Mt. pulcherrima* NSMP and *S. cerevisiae* Eno Syrah were obtained from Lallemand (Montreal, Quebec, Canada). In 2016, Merlot grapes (25.4°Brix, pH 3.50, 4.23 g/L titratable acidity) were crushed and destemmed prior to inoculation with (a) *Mt. pulcherrima* A016, (b) *Mt. pulcherrima* NSMP, or (c) *My. guillermondii* P40D002. After 72 hours, all musts followed by inoculation of *S. cerevisiae* after six days. The control grape musts were those not inoculated with non-*Saccharomyces* yeasts but inoculated at day 0 with *S. cerevisiae*. Soluble solids were monitored with a portable density meter (DMA35, Anton-Paar) while yeast culturabilities were determined using lysine agar (non-*Saccharomyces*) or WL agar (*Saccharomyces*). Cap management consisted of daily punch-downs. At approximately $^1/_3$ reduction of soluble solids, Fermaid-K was added at 24 g/hL. Wines were pressed-off at 0°Brix, racked and moved to 3-4°C after addition of 50 ppm. Additional SO2 was added prior to bottling and storage at 4°C.

Wines inoculated with *Mt. pulcherrima* strains had significantly ($p \leq 0.05$) less ethanol compared to wines fermented with *S. cerevisiae* alone (Table 7). Here, wines inoculated with *Mt. pulcherrima* strains P01A016 and NS-MP contained 13.83 and 13.94% v/v alcohol respectively,

while wines fermented only with *S. cerevisiae* contained 14.86% v/v. However, uninoculated and *My. guillermondii* P40D002 inoculated treatments produced similar concentrations of ethanol compared to the control. Additionally, wines produced with non-*Saccharomyces* yeasts contained similar concentrations or slightly less volatile acidity than those produced solely with *S. cerevisiae*. All yeasts produced similar concentrations of succinic acid and glycerol. These compounds are potential carbon sinks in alternative carbohydrate metabolic pathways, indicating that the fate of the missing carbon from ferments containing *Mt. pulcherrima* is still unknown.

Concentrations of volatile compounds in 2016 Merlot wines were quantified by headspace-solid phase microextraction gas chromatography mass spectroscopy (HS-SPME-GC-MS). Following similar procedures established by Clary et al. (2006), compounds were analyzed with an Agilent HP 6890 fitted with an Agilent HP-5 column (5%-Phenyl-methylsiloxane, 60.0m x 250 µm x 0.25 µm) coupled to a 5973 Mass Selective Detector. Wines produced with non-*Saccharomyces* yeasts contained higher concentrations of total higher alcohols than those produced solely by *S. cerevisiae* (Table 8). Specifically, higher concentrations of 2-methyl-1-propanol (*Mt. pulcherrima* A016, NS-MP, and *My. guillermondii* P40D002), 2&3-methyl-1-butanol (*Mt. pulcherrima* A016) and 2-Phenyethanol (*My. guillermondii* P40D002) compared to *S. cerevisiae* wines are responsible for the increased higher alcohol content. Total ester (excluding ethyl acetate) and volatile acid content did not differ between yeast treatments. However, the uninoculated and the *My. guillermondii* P40D002 treated wines yielded significantly more ethyl acetate (145 and 148 mg/L) that could be perceived negatively by consumers.

In 2017, the experiment was repeated with Merlot grapes (25.5°Brix, pH 3.38, 5.94 g/L titratable acidity), but with only *Mt. pulcherrima* A016 along with the same strain of *Saccharomyces*. Some of the fermentations inoculated solely with *S. cerevisiae* were conducted under aerobic conditions using the same procedure as that with *Mt. pulcherrima*. Wines inoculated with *Mt. pulcherrima* A016 yielded wines containing 14.44% v/v ethanol (Table 9), significantly less than fermentations with only *S. cerevisiae* (15.22 and 15.29% v/v respectively). All wines contained similar concentrations of succinic acid and glycerol. These wines will be bottled and analyzed during 2018 in preparation of chemical and sensory analyses.

Objective 2c: Quantitative assessments of pectinolytic activity were performed spectrophotometrically by quantifying yield of galacturonic acids (Milner and Avignad 1967, Fernández-González et. al. 2004). Yeast strains were grown in 0.67% Yeast Nutrient Base medium (YNB, Difco) supplemented with 1% w/v glucose (JT. Baker), 0.3% w/v tartaric acid, and 0.5% w/v 25,000-50,000 mw polygalacturonic acid (PGA, Alfa Aesar) adjusted to pH 3.5. All yeasts were grown for 3 days at 25°C without agitation. To measure enzymatic activity, 100 µL of yeast culture supernatant was added to 500 µL of 0.1% w/v PGA in 50 mM sodium acetate buffer adjusted to pH 3.5 and incubated for 24 hours at 25°C. The reaction was stopped using the copper reagent. A standard curve was developed using galacturonic acid (GALA) while results were expressed as the µmol GALA released per mL of supernatant (µmol GALA/mL).

Polygalacturonase activity was limited to only certain yeast species and strains (Table 11). Of those tested, 14 of the 30 strains exhibited none to limited (<0.2 µmol GALA/mL) activity against polygalacturonic acid present in the liquid medium. Conversely, high activity (>2 µmol GALA/mL) was observed by *Cr. adeliensis* P44A007, *C. macerans* P41D001, and *K. marxianus*

Ha-63. As such, these strains as well as *P. kluyveri* P01C002 and *I. orientalis* J5-6-2 were selected for further experimentation using grape juice.

For this screening, grape juice (100 mL) was aseptically added milk dilution bottles containing 1 g/L SigmaCel. Sterile-filtered 1% PGA solutions were adjusted to pH 3.6 and then added to the grape juices to achieve a concentration of 0.05%. The non-*Saccharomyces* yeasts were inoculated into the juices as 10^5 cfu/mL followed by *S. cerevisiae* after 48 hours.

All treatments yielded similar amounts of polygalacturonic acid residues with the exceptions of *C. macerans* and *K. marxianus* (Table 12) while not producing noticeable aroma faults. Grape juice with *Cr. adeliensis* contained the highest concentration of pectin breakdown although exhibited poor survivability in grape must. While *I. orientalis* and *P. kluyveri* produced less pectin breakdown, these strains survived longer. To limit the potential problem of survivability, a mixed starter containing *Cr. adeliensis, I. orientalis,* and *P. kluyveri* was selected to increase both initial and long-term enzyme production during winemaking trials in 2016-2017.

In 2016, wines were produced in larger fermentations (200L) using Merlot grapes. Here, a "cocktail" of three species of non-*Saccharomyces* yeasts with high demonstrated pectinase activities (*Issatchenkia orientalis* J5-6-2, *Pichia kluyveri* P01C002, and *Cryptococcus adeliensis* P44A007) were inoculated into musts six days prior to addition of *S. cerevisiae* to complete alcoholic fermentation. At the time of writing, these wines have not yet been bottled but preliminary sensory and e-tongue analyses are being conducted (spring to summer 2018) to determine if there are any changes to mouthfeel due to the presence of these yeasts.

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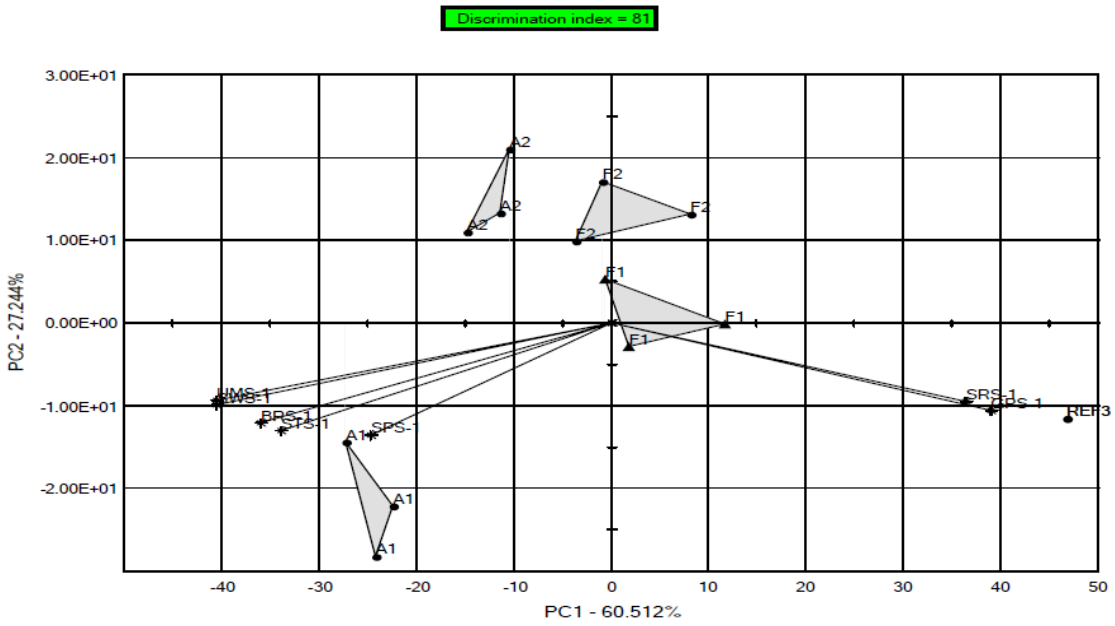

Figure 1. Discriminant analysis from electronic tongue data showing differences in taste from wines aged in low toasted American (A) or French (F) barrels infected with *B. bruxellensis* strain E1 (1) or I1a (2) after 3 months.

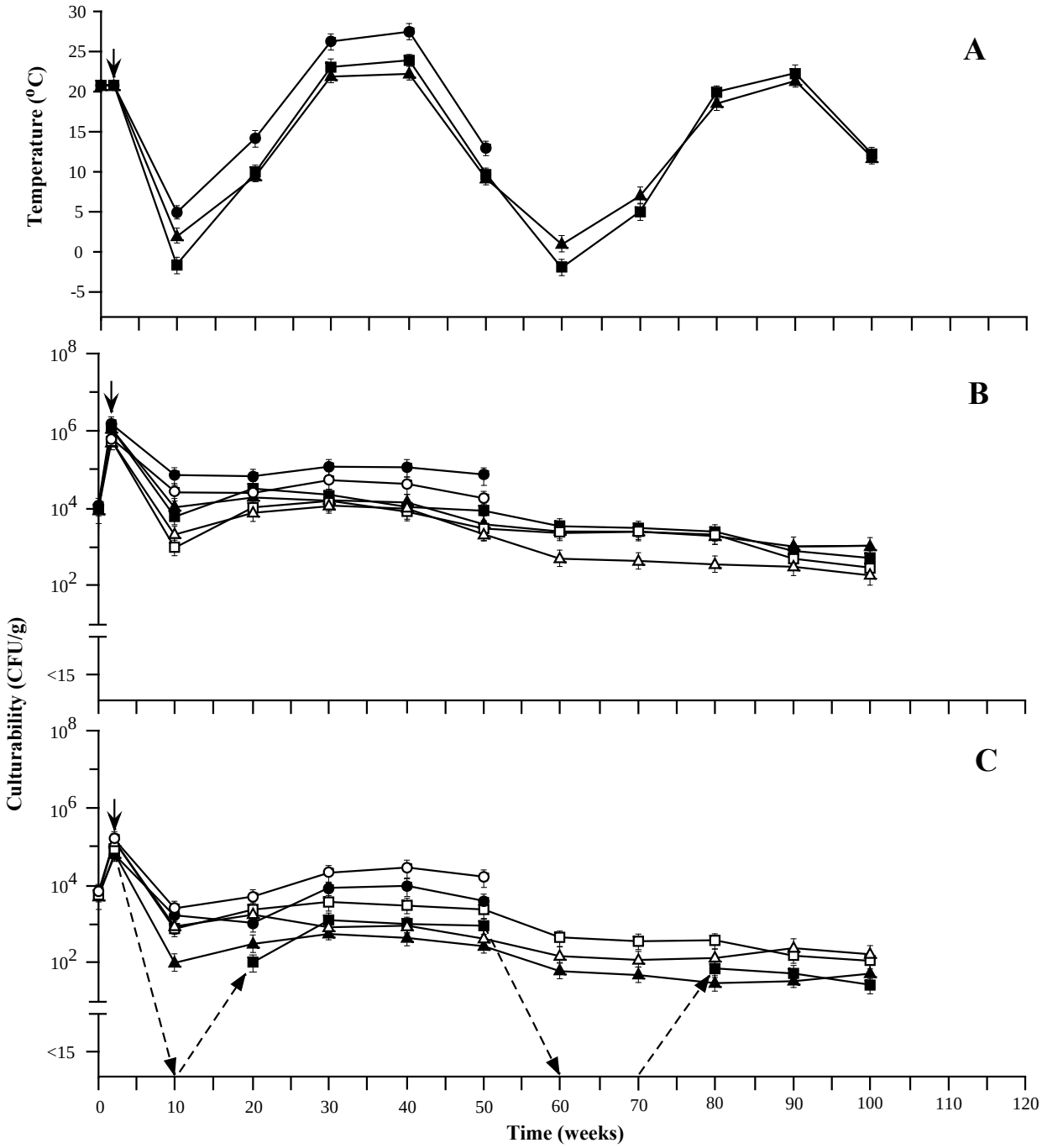

Figure 2. Culturability of *B. bruxellensis* strain I1a in Syrah grape pomace either early press (▲, ■, ●) or completely fermented (△, □, ○) and then sterilized (A) or not (B) before transfer to vineyard 1 (▲,△) vineyard 2 (■,□), or vineyard 3 (●,○).

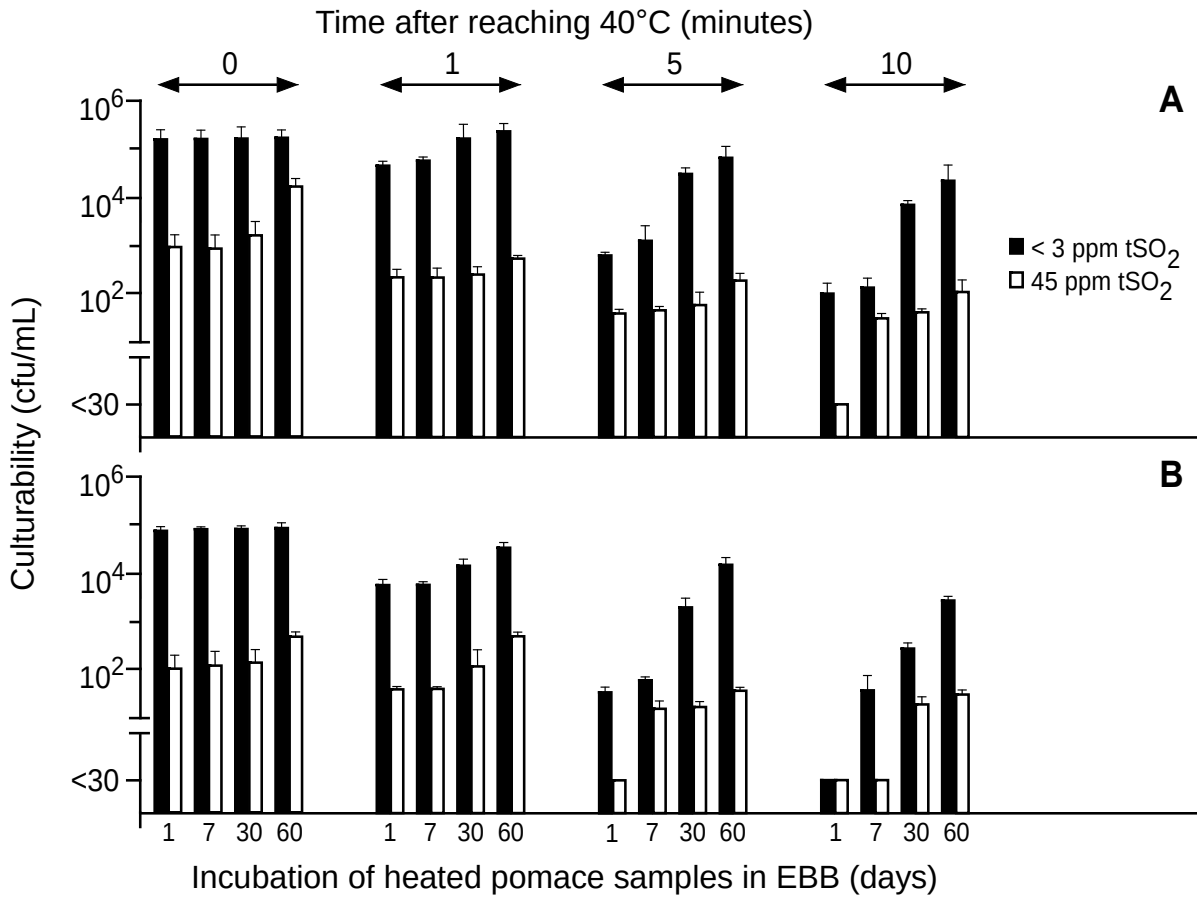

Figure 3. *B. bruxellensis* recovered from pomace samples containing 11.7% (**A**) or 15.1% (**B**) v/v ethanol and <3 (■) or 45 (□) ppm tSO2. After the center of 100g pomace samples reached 40°C, they were held at that temperature for 0, 1, 5, or 10 min prior to placement in a *Brettanomyces* enrichment medium (EBB) for up to 60 days.

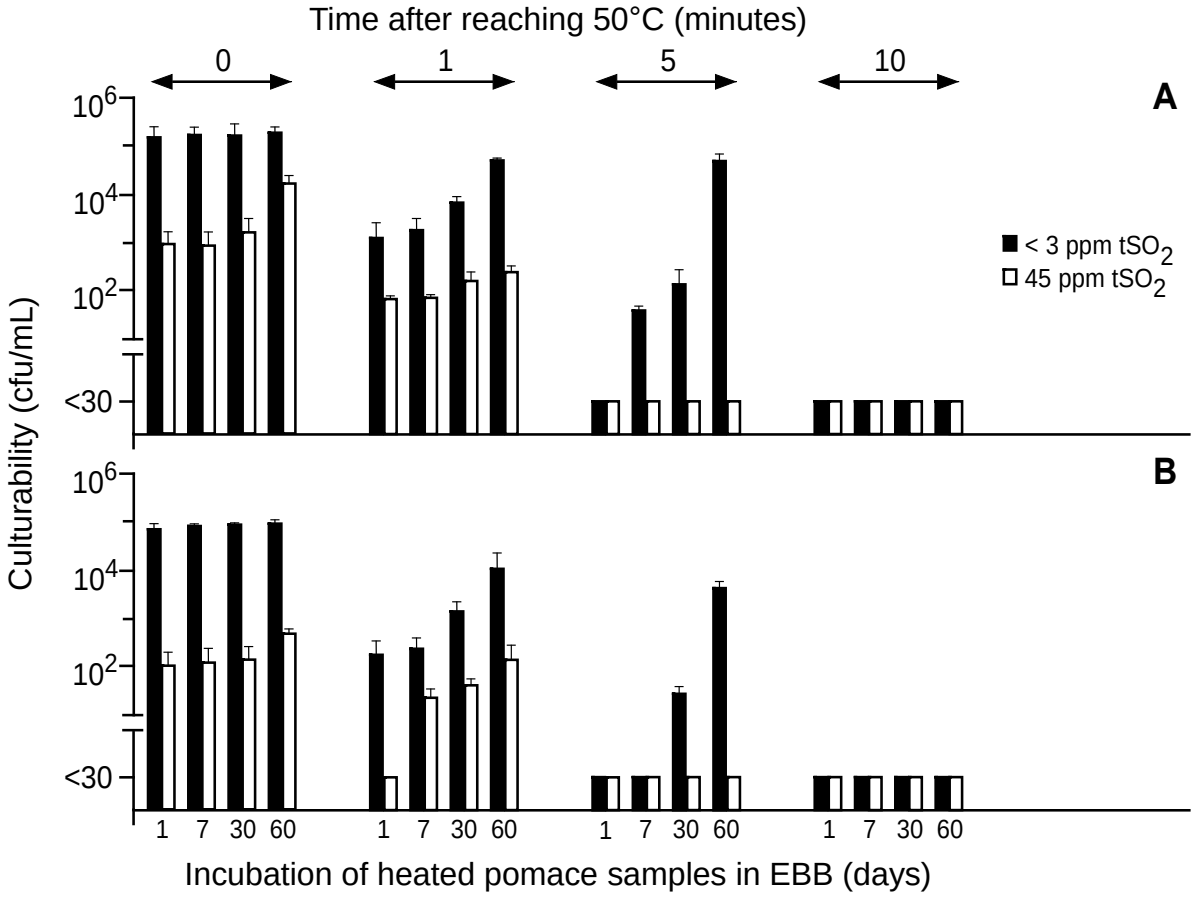

Figure 4. *B. bruxellensis* recovered from pomace samples containing 11.7% (**A**) or 15.1% (**B**) v/v ethanol and <3 (■) or 45 (□) ppm tSO2. After the center of 100g pomace samples reached 50°C, they were held at that temperature for 0, 1, 5, or 10 min prior to placement in a *Brettanomyces* enrichment medium (EBB) for up to 60 days.

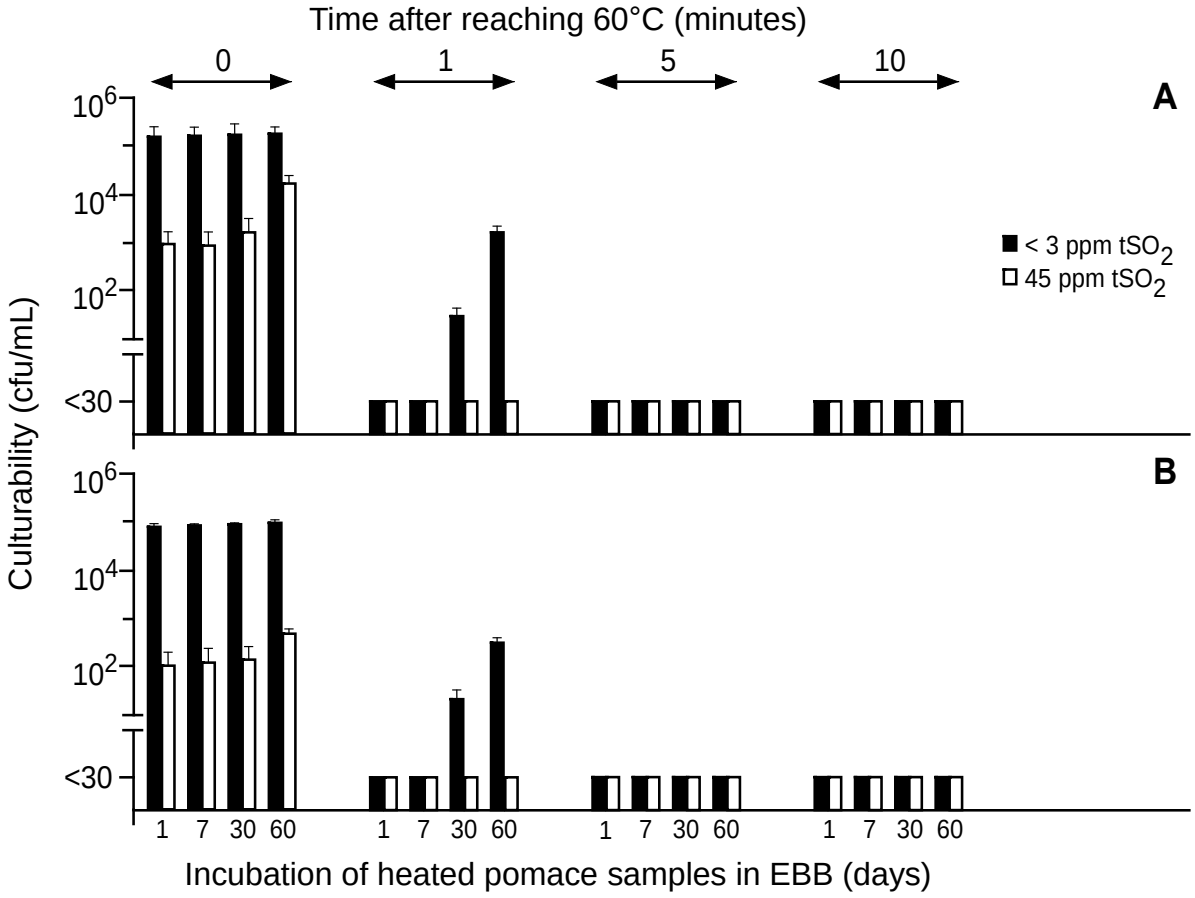

Figure 5. *B. bruxellensis* recovered from pomace samples containing 11.7% (**A**) or 15.1% (**B**) v/v ethanol and <3 (■) or 45 (□) ppm tSO2. After the center of 100g pomace samples reached 60°C, they were held at that temperature for 0, 1, 5, or 10 min prior to placement in a *Brettanomyces* enrichment medium (EBB) for up to 60 days.

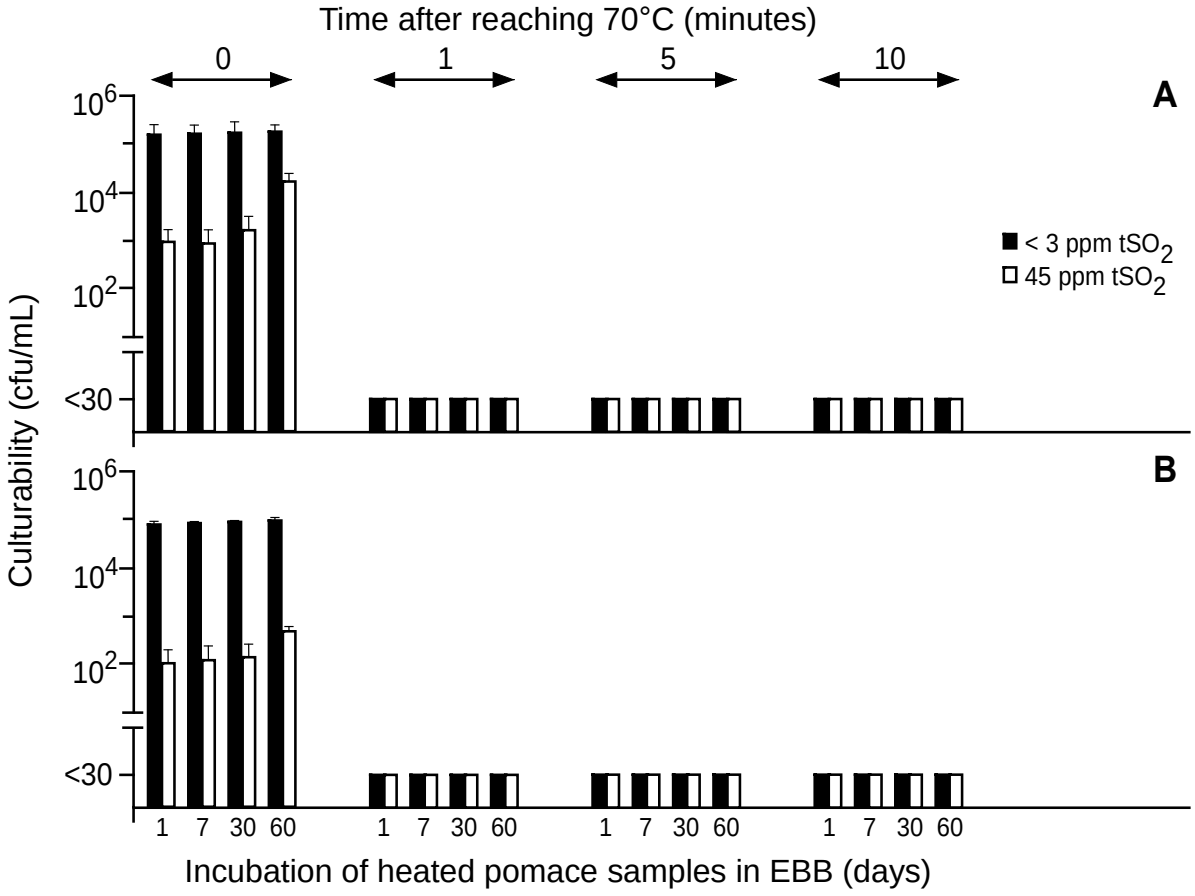

Figure 6. *B. bruxellensis* recovered from pomace samples containing 11.7% (**A**) or 15.1% (**B**) v/v ethanol and <3 (■) or 45 (□) ppm tSO2. After the center of 100g pomace samples reached 70°C, they were held at that temperature for 0, 1, 5, or 10 min prior to placement in a *Brettanomyces* enrichment medium (EBB) for up to 60 days.

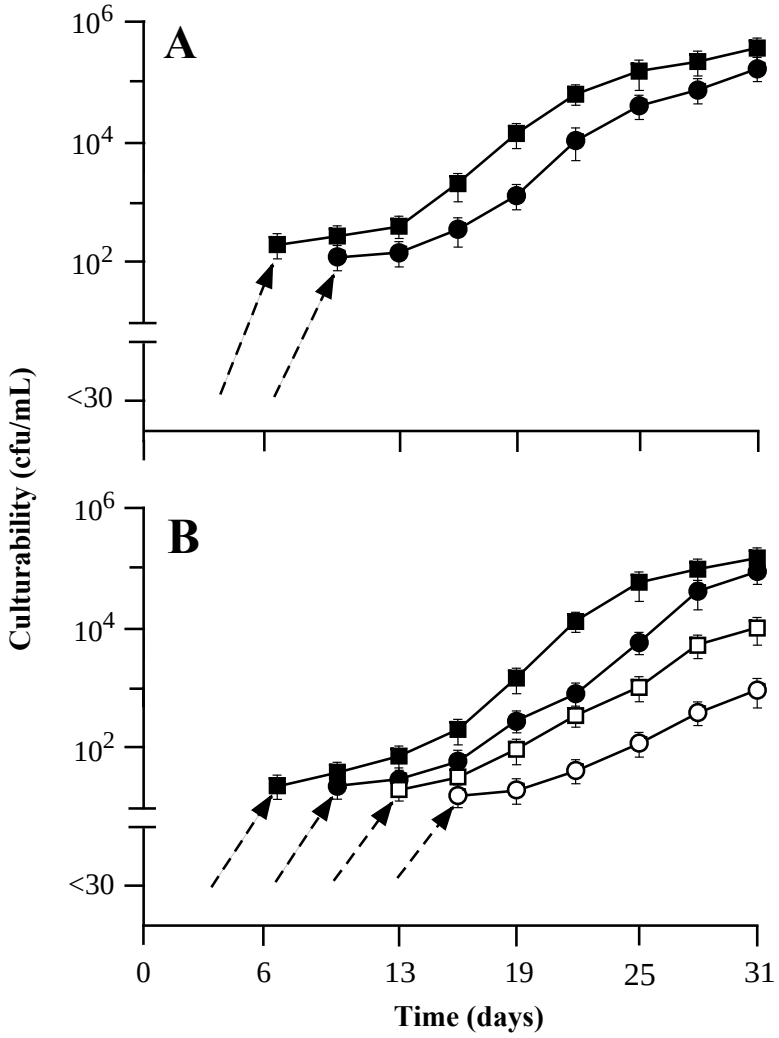

Figure 7. Culturability of *B. bruxellensis* from three-year-old American medium heavy (●, ○) or French medium light (■, □) toasted 225 L industry barrels. Staves located at the top (A) or bottom (B) of the barrels were sawn into cross-sections that represent depths of 0 to 4 mm (●, ■) or 5 to 9 mm (○, □) from inside-to-outside.

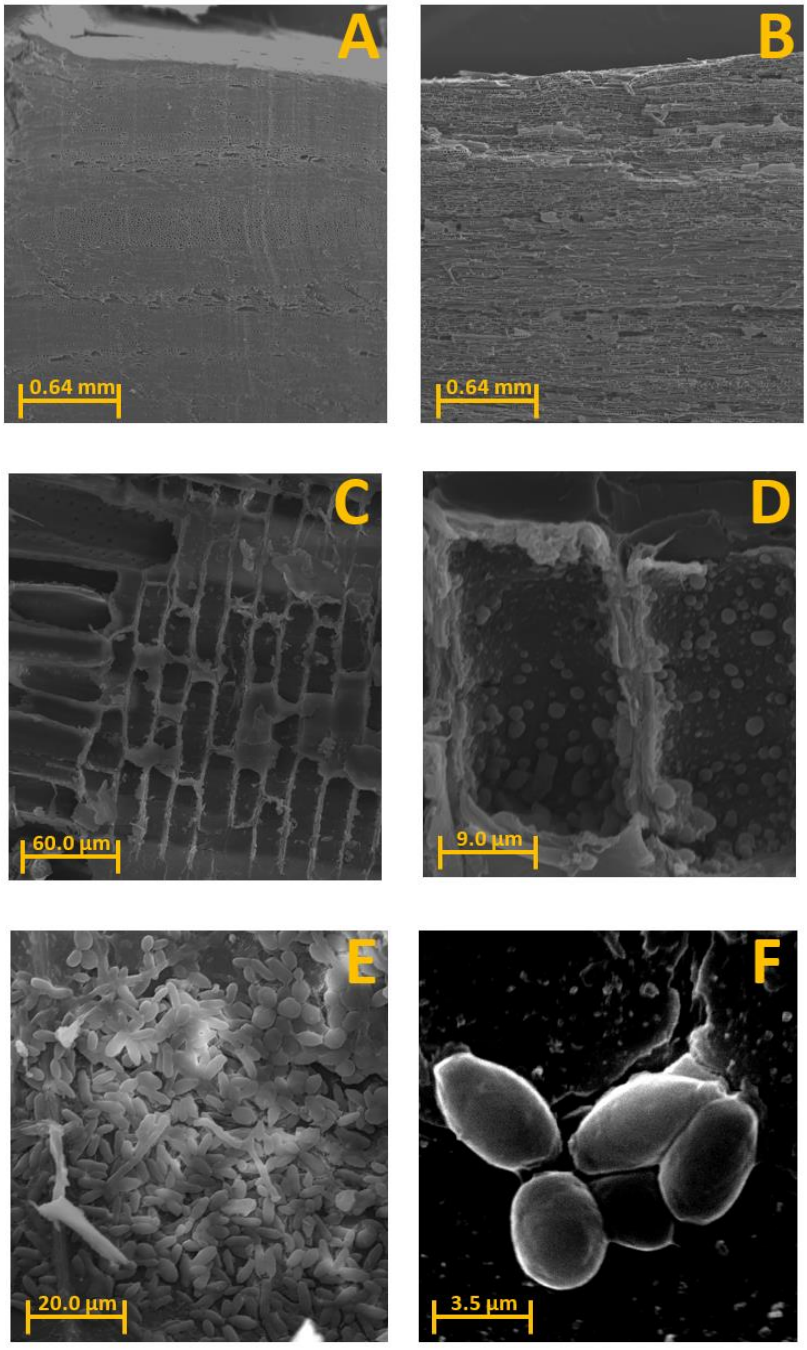

Figure 8. Electron microscopy images from infected oak barrels including longitudinal cross sections of American light toasted (A) and French heavy toasted (B) oak staves, enlarged xylem vessels (C, D), observation of possible pseudohyphae structures (E) and suspected *B. bruxellensis* found at a 6-8 mm depth in a heavy toasted French oak stave.

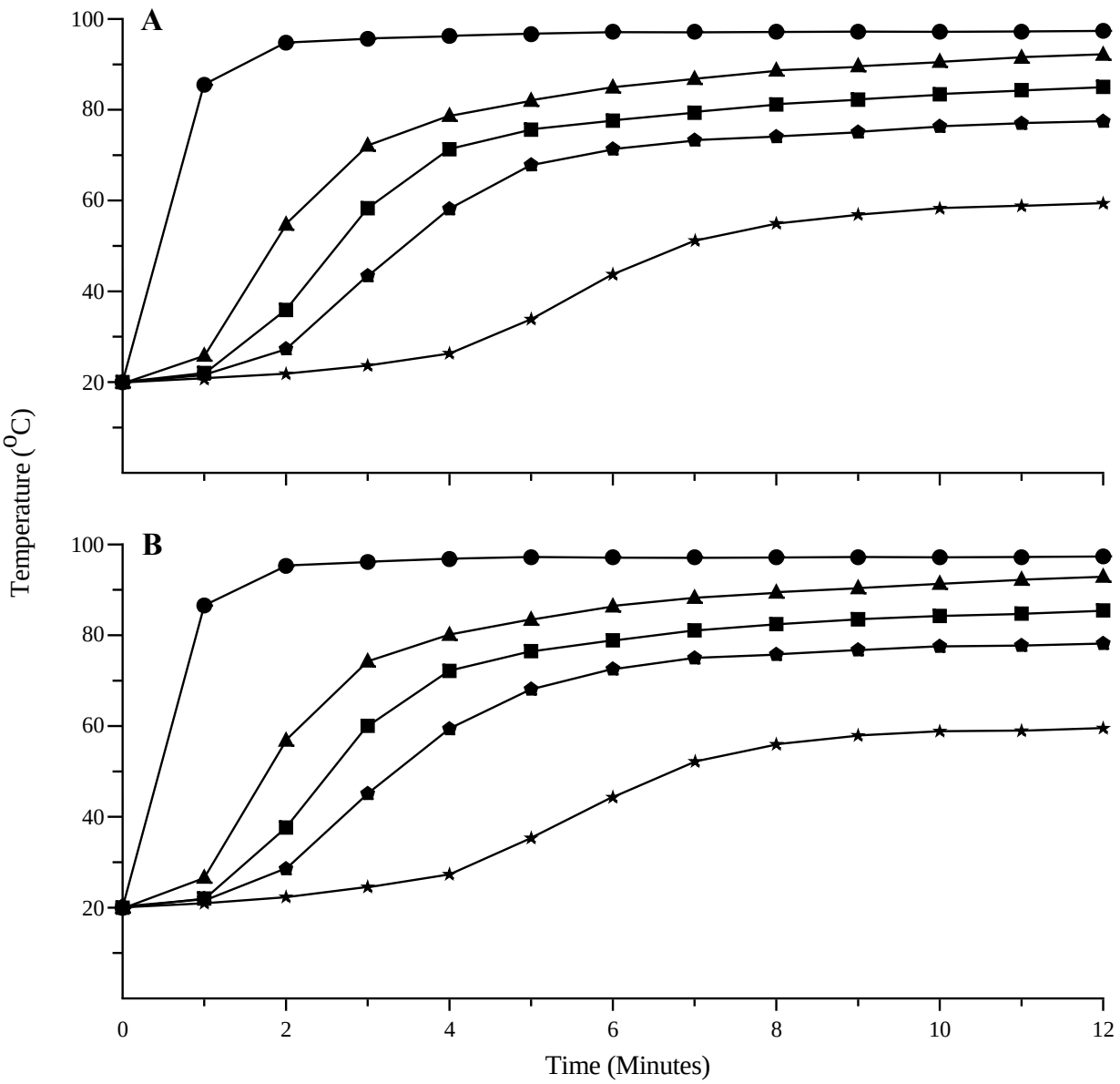

Figure 9. Temperature changes during steaming of American medium-high toasted (A) or French medium-low toasted (B) oak staves at depths of 0 (●), 4.5 (▲), 9.5 (■), 14.5 (⬟) and 25 (★) mm.

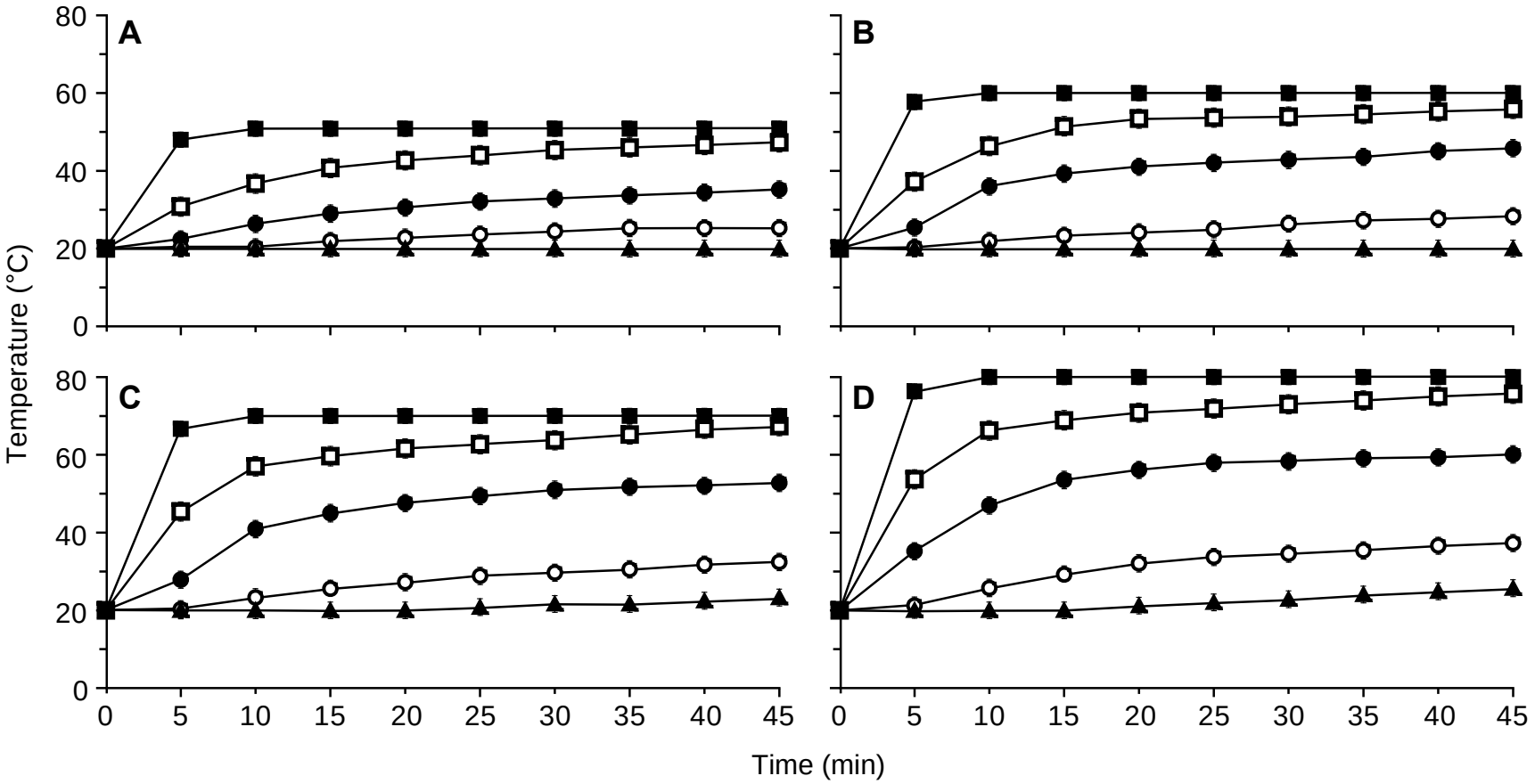

Figure 10. Temperature changes to 3 x 3 oak blocks obtained from three-year-old 225 L barrel staves placed in hot water at 50° (**A**), 60° (**B**), 70° (**C**) or 80°C (**D**). Thermocouples were placed in the blocks at depths of 0 (●), 4.5 (▲), 9.5 (■), 14.5 (⬟) and 25 (★) mm.

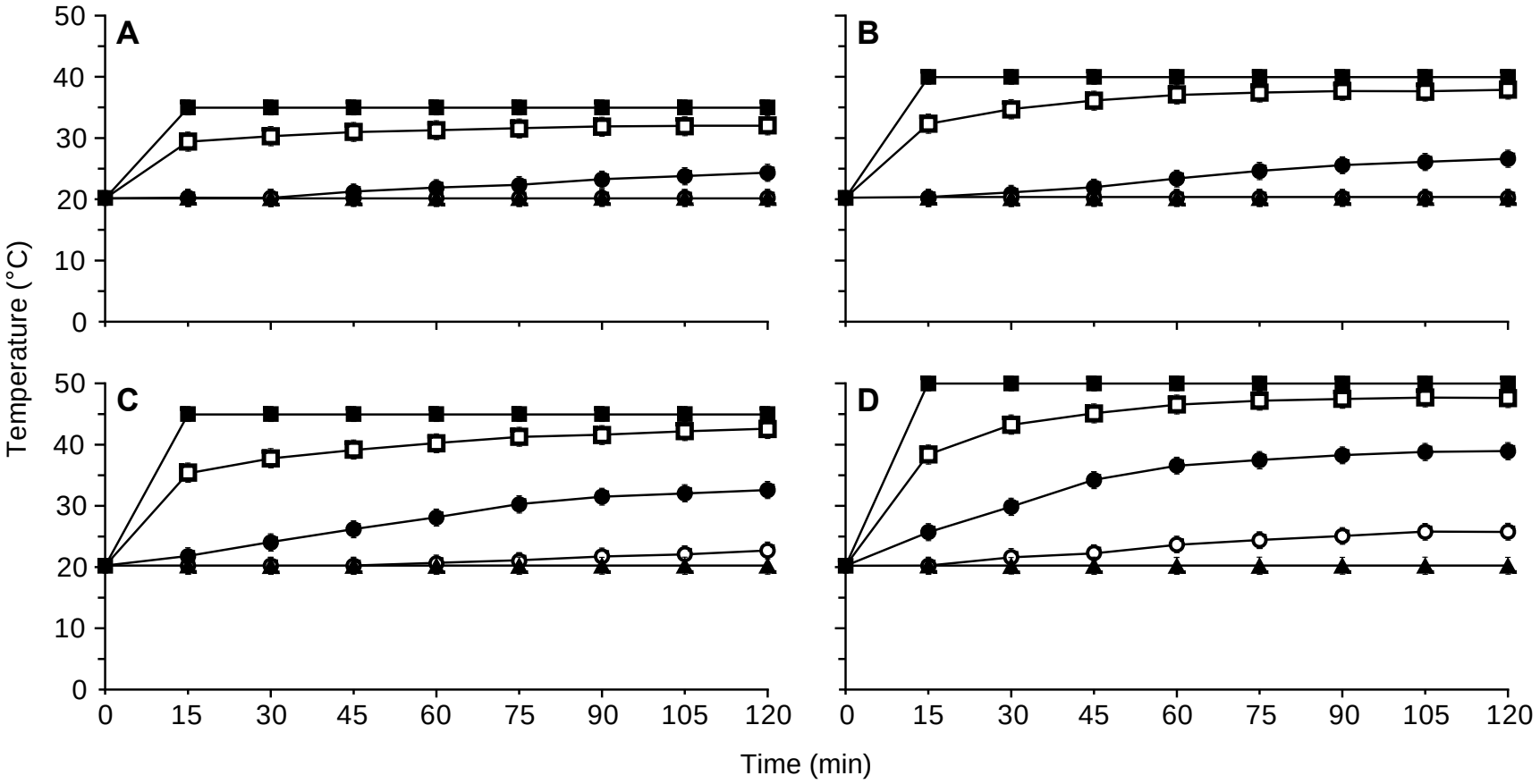

Figure 11. Temperature changes to 3 x 3 oak blocks obtained from three-year-old 225 L barrel staves placed in warm wine at 35° (**A**), 40° (**B**), 45° (**C**) or 50°C (**D**). Thermocouples were placed in the blocks at depths of 0 (●), 4.5 (▲), 9.5 (■), 14.5 (⬟) and 25 (★) mm.

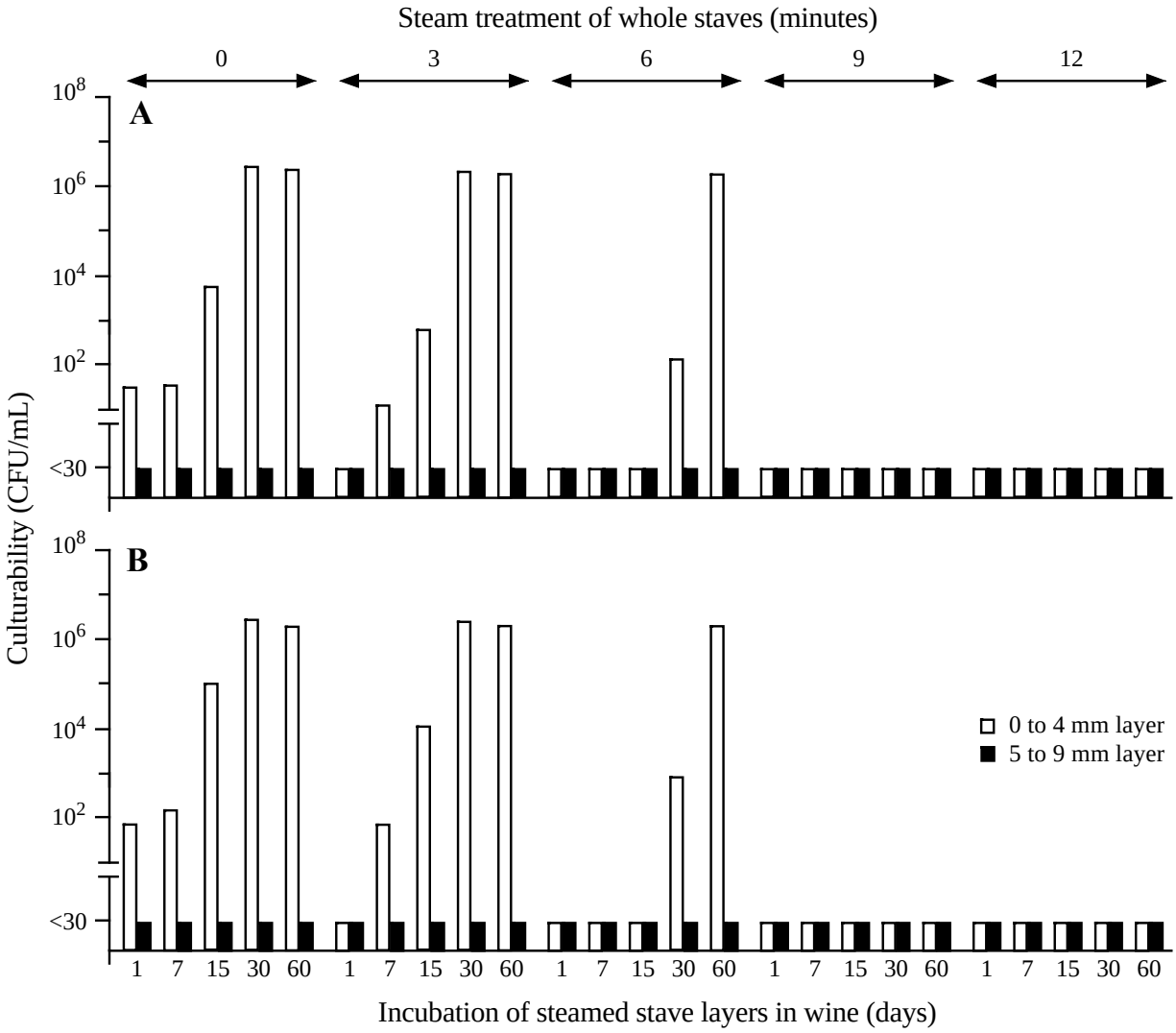

Figure 12. Recovery of *B. bruxellensis* strain I1a from American lightly toasted (A) or American heavily toasted (B) oak staves (16 L barrels) steamed for up to 12 min.

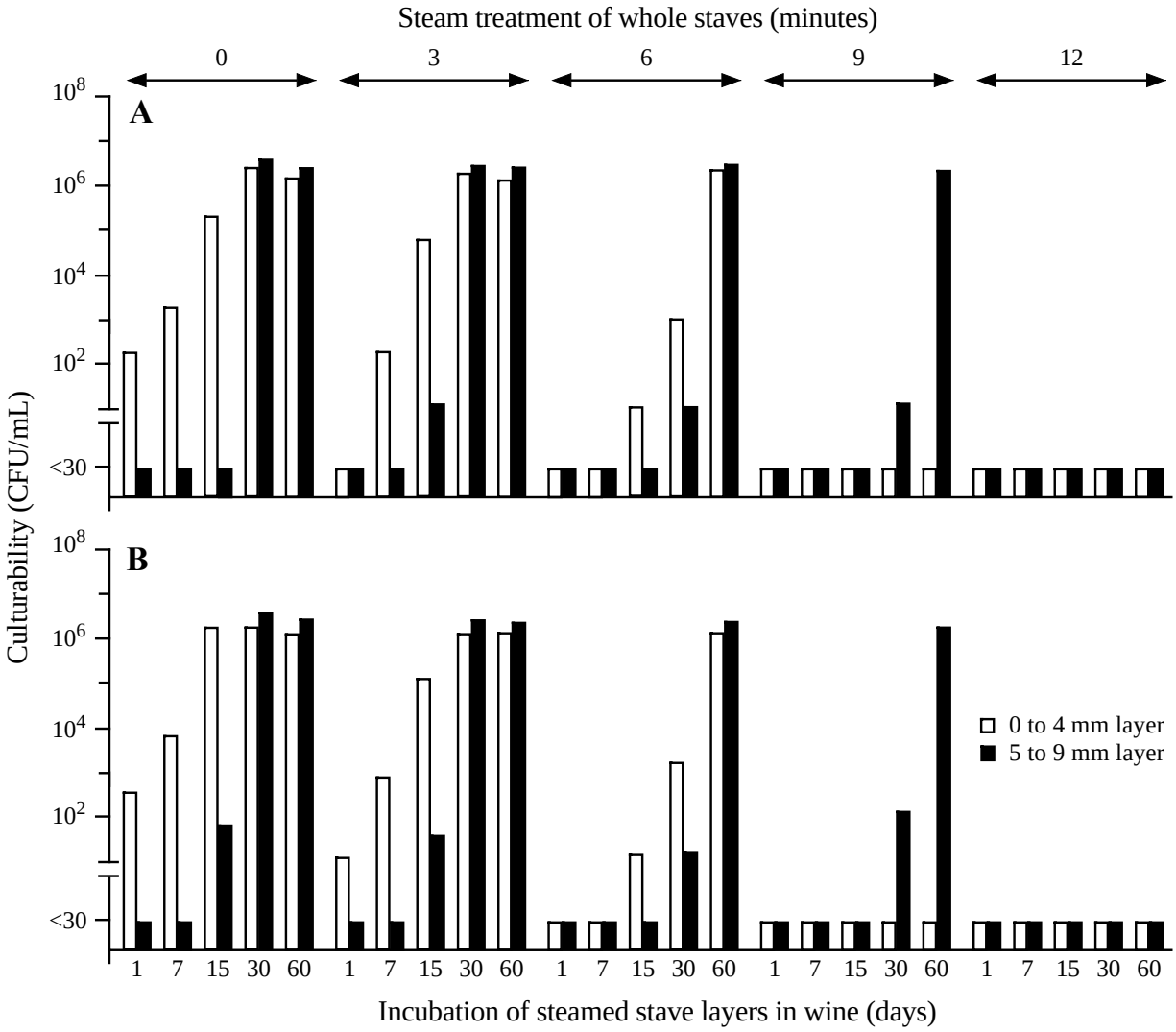

Figure 13. Recovery of *B. bruxellensis* strain I1a from French lightly toasted (A) or French heavily toasted (B) oak staves (16 L barrels) steamed for up to 12 min.

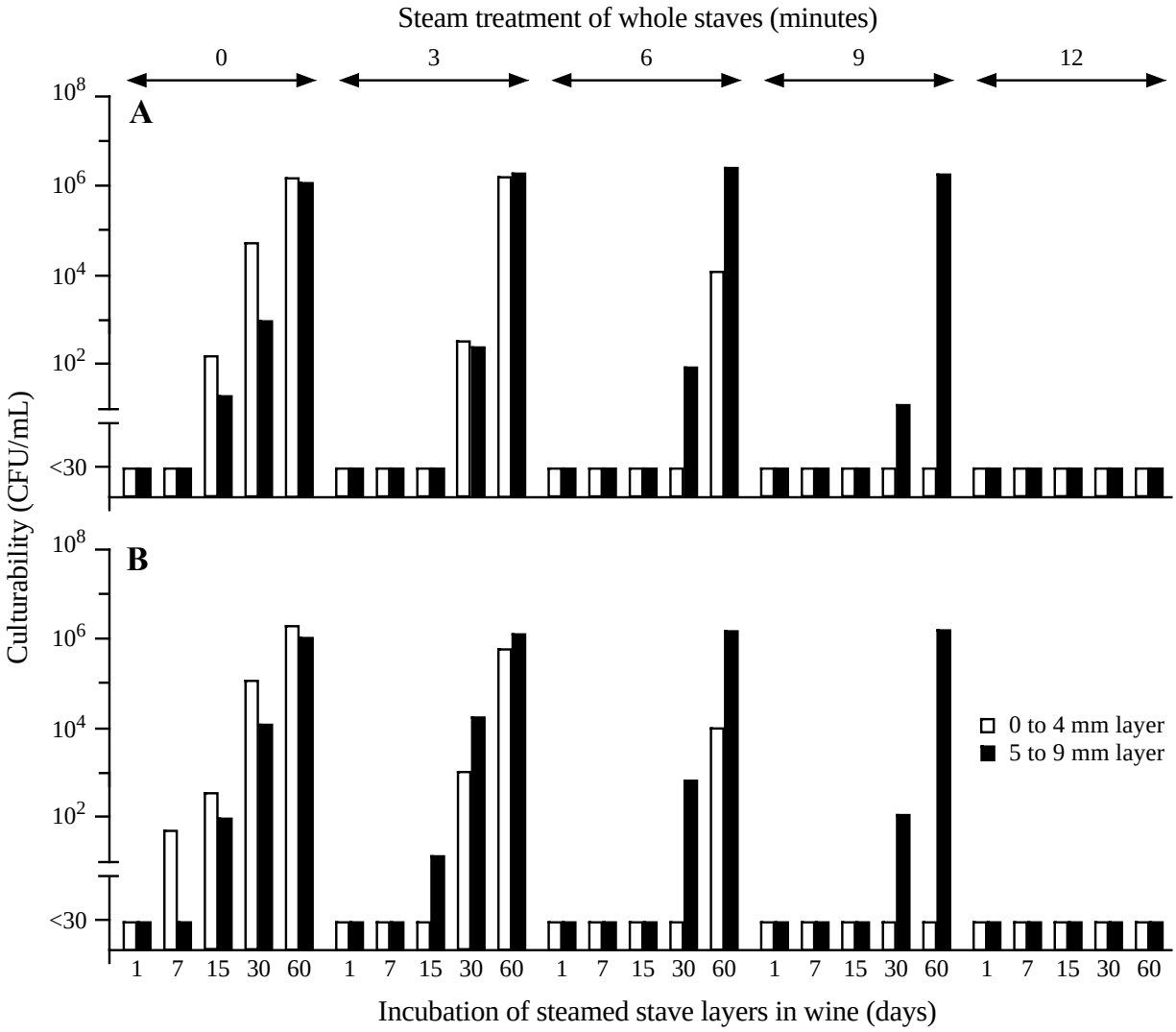

Figure 14. Recovery of *B. bruxellensis* from American medium-high toast (A) or French (B) medium-low oak staves (225 L barrels) steamed for up to 12 min.

Table 1. Populations of *B. bruxellensis* recovered from different depths from oak staves by analyzing shavings prepared using a Forstner drill bit.

Barrel	Toast Level	Pigment Penetration (mm)	Stave Layer (mm)	Population (cfu/mm^3)
16-L (American)	Low	3.5	0 to 2	4130
			2 to 4	535
			4 to 6	*
			6 to 8	*
16-L (American)	High	3.8	0 to 2	4510
			2 to 4	693
			4 to 6	*
			6 to 8	*
16-L (French)	Low	4.6	0 to 2	5610
			2 to 4	901
			4 to 6	123
			6 to 8	*
16-L (French)	High	4.7	0 to 2	6400
			2 to 4	1043
			4 to 6	136
			6 to 8	10
225-L (American)	Medium-high	4.3	0 to 2	56
			2 to 4	193
			4 to 6	40
			6 to 8	*
225-L (French)	Medium-low	5.2	0 to 2	71
			2 to 4	439
			4 to 6	201
			6 to 8	119

*Below the limit of detection after incubation in EBB enhancment medium (EBB) for >30 days.

Table 2. Impacts of warm water or wine on oak barrel staves infected with *B. bruxellensis*.

Treatment	Temperature (°C)	Minimum Treatment Time†	
		Stave depth (0 to 4 mm)	Stave depth (5 to 9 mm)
Water	50°	60	>90
	60°	30	45
	70°	20	30
	80°	15	20
Wine A*	35°	>120	>120
	40°	>120	>120
	45°	90	>120
	50°	60	120
Wine B*	35°	>120	>120
	40°	120	>120
	45°	75	>120
	50°	45	120

†Minimum treatment time required to not recover viable cells.

*Composition of the red wines were wine A (11% v/v ethanol, pH 3.5, 35 mg/L total SO_2, and 12 mg/L free SO_2) and wine B (15% v/v ethanol, pH 3.5, 37 mg/L total SO_2, and 14 mg/L free SO_2).

Table 3. Sugar and nitrogen consumption, ethanol yield, and growth rate of yeasts in grape juice* without aeration before subsequent inoculation of *Saccharomyces cerevisiae*.

Species	Glucose (g/L)	Fructose (g/L)	Amino N (mg/L)	Ammonia N (mg/L)	Ethanol (g/g sugar consumed)	Doubling Time (hr)
Ca. californica	50.2	34.5	53	120	0.12	5.4
Ca. oleophila	36.1	16.6	41	150	0.29	4.7
Ca. railenensis	39.9	23.7	37	110	0.18	5.5
Cr. adelienses	20.7	17.3	16	40	0.01	6.8
Cr. saitoi	34.4	31.1	28	14	0.00	7.0
Cu. pallidacorallinum	20.7	17.3	16	40	0.01	7.1
H. uvarum	50.9	58.3	31	44	0.22	5.3
I. orientalis	62.0	14.3	44	9	0.26	4.6
K. marxianus	89.6	39.9	40	80	0.24	6.6
Mt. chrysoperlae	64.2	47.6	41	47	0.06	5.7
Mt. pulcherrima	32.0	20.0	69	78	0.30	4.9
My. carribica	23.9	8.5	46	54	0.43	5.6
My. guillermondii	34.1	27.8	34	66	0.11	6.6
P. fermentans	48.3	14.7	26	12	0.23	4.4
P. kluyveri	57.4	25.8	31	47	0.14	5.1
P. membranifaciens	124.4	85.1	100	138	0.25	7.3
S. cerevisiae	160	117.7	121	150	0.25	4.0
W. anomalus	38.3	10.8	50	74	0.24	4.5
Y. mexicana	58.9	47.5	31	91	0.10	8.8

*Grape juice initially contained 155 g/L of each sugar (total 310 g/L), 150 mg/L amino nitrogen (N) and 150 mg/L ammonium (N).

Table 4. Sugar and nitrogen consumption, ethanol yield, and growth rate of yeasts in grape juice* with aeration before subsequent inoculation of *Saccharomyces cerevisiae*.

Species	Glucose (g/L)	Fructose (g/L)	Amino N (mg/L)	Ammonia N (mg/L)	Ethanol (g/g sugar consumed)	Doubling Time (hr)
Ca. californica	42.1	20.2	114	141	0.15	9.9
Ca. oleophila	44.0	26.6	112	48	0.12	4.8
Ca. railenensis	33.0	14.2	111	138	0.16	6.3
Cr. adelienses	34.4	21.9	108	144	0.01	5.0
Cr. saitoi	51.4	38.1	112	146	0.00	6.0
Cu. pallidacorallinum	34.4	21.9	108	144	0.01	5.3
H. uvarum	27.4	23.8	56	84	0.09	13.9
I. orientalis	40.0	9.1	105	10	0.10	4.9
K. marxianus	35.8	21.5	55	72	0.05	13.0
Mt. chrysoperlae	59.3	47.2	109	122	0.11	11.4
Mt. pulcherrima	38.0	20.8	98	95	0.33	4.9
My. carribica	36.2	12.2	87	92	0.17	6.9
My. guillermondii	72.6	49.7	84	127	0.06	5.9
P. fermentans	33.7	7.5	87	82	0.06	4.4
P. kluyveri	39.6	28.1	113	124	0.06	9.1
P. membranifaciens	62.3	47.3	129	150	0.06	14.8
S. cerevisiae	35.5	15.3	32	58	0.16	4.9
W. anomalus	59.6	17.0	126	136	0.20	4.8
Y. mexicana	65.4	54.2	76	150	0.03	7.0

*Grape juice initially contained 155 g/L of each sugar (total 310 g/L), 150 mg/L amino nitrogen (N), and 150 mg/L ammonium nitrogen (N).

Table 5. Production of ethanol and acetic acid as well as sugar consumption after fermentation of grape juices* without aeration before subsequent inoculation of *Saccharomyces cerevisiae*.

Species	Ethanol (% v/v)	Acetic Acid (g/L)	Total Sugar Consumed (%)
Ca. californica	15.7	1.44	97.9
Ca. oleophila	15.0	1.38	97.4
Ca. railenensis	15.8	1.07	98.5
Cr. adelienses	15.9	0.86	98.7
Cr. saitoi	16.1	1.05	99.5
Cu. pallidacorallinum	16.0	0.73	99.5
H. uvarum	16.1	1.39	99.7
I. orientalis	16.1	1.43	99.2
K. marxianus	14.6	1.19	92.7
Mt. chrysoperlae	15.2	0.71	93.8
Mt. pulcherrima	15.7	0.70	96.2
My. carribica	16.1	0.80	94.6
My. guillermondii	15.8	0.86	99.6
P. fermentans	16.4	1.03	99.8
P. kluyveri	15.8	1.14	99.8
P. membranifaciens	16.0	1.00	98.5
S. cerevisiae	16.1	0.62	97.5
W. anomalus	14.7	1.28	95.3
Y. mexicana	16.0	0.78	98.7

*Grape juice initially contained 155 g/L of each sugar (total 310 g/L), 150 mg/L amino nitrogen(N) and 150 mg/L ammonium (N).

Table 6. Production of ethanol and acetic acid as well as sugar consumption after fermentation of grape juices* with aeration before subsequent inoculation of *Saccharomyces cerevisiae*.

Species	Ethanol (% v/v)	Acetic Acid (g/L)	Total Sugar Consumed (%)
Ca. californica	14.2	1.77	96.6
Ca. oleophila	14.4	1.98	93.8
Ca. railenensis	14.5	1.92	95.1
Cr. adelienses	14.6	1.48	97.4
Cr. saitoi	14.1	1.68	93.6
Cu. pallidacorallinum	13.7	1.15	98.1
H. uvarum	14.7	1.80	99.2
I. orientalis	15.9	1.47	98.1
K. marxianus	12.4	2.52	97.4
Mt. chrysoperlae	14.5	1.68	96.9
Mt. pulcherrima	15.4	0.92	96.0
My. carribica	15.5	1.12	96.3
My. guillermondii	14.2	1.53	98.0
P. fermentans	15.0	1.49	87.5
P. kluyveri	13.8	2.05	96.0
P. membranifaciens	13.6	2.47	97.5
S. cerevisiae	15.9	1.45	99.5
W. anomalus	13.4	2.40	96.6
Y. mexicana	15.4	1.38	98.9

*Grape juice initially contained 155 g/L of each sugar (total 310 g/L), 150 mg/L amino nitrogen(N) and 150 mg/L ammonium (N).

Table 7. Composition of Merlot wines from grape juice concentrate and fermented in open-top stainless steel tanks. Ferments were sequentially inoculated with non-*Saccharomyces* yeasts followed by *S. cerevisiae*.

Species/Strain	Ethanol (% v/v)	Titratable acidity (g/L)	Volatile acidity (g/L)
Mt. chrysoperlae P40A002	13.3^{e}	7.20^{a}	0.51^{b}
Mt. pulcherrima P01A016	11.7^{a}	9.09^{d}	0.23^{a}
Mt. pulcherrima NS-MP*	12.1^{b}	8.71^{cd}	0.25^{a}
Mt. fructicola NS-MF*	12.1^{bc}	8.54^{c}	0.26^{a}
My. guillermondii P40D002	12.3^{c}	8.25^{bc}	0.24^{a}
P. kluyveri P01C002	13.3^{e}	7.74^{ab}	0.33^{a}
P. membranifaciens P43C010	13.0^{d}	7.66^{a}	0.25^{a}
S. cerevisiae ICV D254*	13.7^{f}	7.87^{ab}	0.60^{bc}
T. delbrueckii NS-TD*	13.4^{e}	7.83^{ab}	0.62^{c}

* Strains provided by Lallemand, Inc. (Montréal, Quebec, Canada).
Means within a column with different superscripts are statistically significant at $p \leq 0.05$.

Table 8. Chemical composition of dry wines produced inoculation of a combination of non-*Saccharomyces/S. cerevisiae* or by *S. cerevisiae* alone.

Species/Strain	Ethanol (% v/v)	pH	Titratable acidity (g/L)	Volatile acidity (g/L)	Succinic acid (g/L)	Glycerol (g/L)
Mt. pulcherrima P01A016	13.83[a]	3.23[a]	6.09[b]	0.35[ab]	1.91[a]	10.10[a]
Mt. pulcherrima NS-MP	13.94[a]	3.21[b]	6.09[b]	0.33[a]	1.74[a]	10.07[a]
My. guillermondii P40D002	15.01[b]	3.29[cd]	6.81[c]	0.40[c]	1.82[a]	10.06[a]
S. cerevisiae Eno. Syrah	14.86[b]	3.31[cd]	5.56[a]	0.37[bc]	1.75[a]	9.99[a]

[a-d] Means values within columns with different superscripts are significantly different ($p \leq 0.05$) using Tukey's HSD.

Table 9. Concentrations (mg/L) of volatile components present in 2016 Merlot wines produced by inoculation of a combination of non-*Saccharomyces/S. cerevisiae* or by *S. cerevisiae* alone.

Compound	*Mt. pulcherrima* P01A016	*Mt. pulcherrima* NS-MP	*My. guillermondii* P40D002	*S. cerevisiae* Eno. Syrah
1-Propanol	5.33 [a]	3.36 [a]	4.60 [a]	2.52 [a]
2-Methyl-1-propanol	61.4 [b]	64.5 [b]	70.4 [b]	29.0 [a]
2 (&3)-Methyl-1-butanol	136 [ab]	165 [b]	144 [ab]	108 [a]
1-Hexanol	0.98 [a]	0.95 [a]	1.18 [a]	1.18 [ab]
1-Octanol	1.52 [a]	0.72 [a]	1.23 [a]	1.84 [a]
2-Phenylethanol	37.9 [a]	37.8 [a]	45.3 [b]	36.5 [a]
Ethyl acetate	73.1 [a]	64.1 [a]	148 [b]	52.3 [a]
2(&3)-Methyl butyl acetate	0.81 [ab]	0.86 [ab]	1.02 [b]	0.71 [a]
Hexyl acetate	0.01 [a]	0.01 [a]	0.01 [a]	0.01 [a]
Diethyl succinate	0.99 [ab]	1.00 [ab]	1.05 [ab]	1.24 [b]
2-Phenylethyl acetate	0.04 [ab]	0.05 [bc]	0.07 [c]	0.02 [a]
Ethyl butanoate	0.19 [a]	0.19 [a]	0.19 [a]	0.22 [a]
Ethyl hexanoate	0.07 [a]	0.04 [a]	0.04 [a]	0.09 [a]
Ethyl octanoate	0.39 [ab]	0.35 [a]	0.35 [a]	0.54 [b]
Hexanoic acid	3.52 [ab]	5.27 [c]	2.87 [a]	4.90 [bc]
Octanoic acid	3.39 [a]	3.66 [a]	2.74 [a]	4.29 [a]

[a-c] Mean values within rows with different superscripts are significantly different ($p \leq 0.05$) using Tukey's HSD.

Table 10. Chemical composition of 2017 Merlot wines produced by inoculation of a combination of non-*Saccharomyces/S. cerevisiae* or by *S. cerevisiae* alone.

Species/Strain	Ethanol (% v/v)	pH	Titratable acidity (g/L)	Volatile acidity (g/L)	Succinic acid (g/L)	Glycerol (g/L)
Mt. pulcherrima P01A016	14.44[a]	3.41[a]	7.23[b]	0.60[a]	2.77[a]	10.15[a]
S. cerevisiae (aerobic)*	15.22[b]	3.45[ab]	6.80[ab]	0.60[a]	3.09[b]	9.98[a]
S. cerevisiae	15.29[b]	3.48[b]	6.70[a]	0.68[b]	3.06[b]	9.94[a]

* Some fermentations inoculated only with *S. cerevisiae* were conducted under aerobic conditions for three days, similar to ferments with *M. pulcherrima* A016.

[a-b] Means values within columns with different superscripts are significantly different ($p \leq 0.05$) using Tukey's HSD.

Table 11. Polygalacturonase activity of various yeasts grown in liquid media.

Species	Strain	Galacturonic acid released (µmole/mL)	Species	Strain	Galacturonic acid released (µmole/mL)
Ca. californica	P01C003	0.70	*Mt. chrysoperlae*	P34B007	0.32
Cr. adelienses	P02B003	0.67	*Mt. fructicola*	NS-MF***	0.41
	P25A001	0.00	*Mt. pulcherrima*	NS-MP***	0.10
	P42A008	0.79		P01A006	0.00
	P42C006	0.12		P44A006	0.01
	P44A007	4.06	*P. fermentans*	M1-3-1*	0.27
Ca. oleophila	P40C006	0.28	*P. kluyveri*	J5-6-2*	0.43
Ca. railenensis	RCAA002	0.11		P01C002	0.96
Cr. macerans	P41D001	4.36		P43C009	0.69
Cr. saitoi	P01D003	0.00	*Rh. colostri*	P42A002	0.00
	P40D003	0.00		P42C002	0.24
H. uvarum	RCHB002	0.00	*S. cerevisiae*	ICV D254***	0.00
I. orientalis	J5-6-5*	1.24	*T. delbrueckii*	NS-TD***	0.00
K. marxianus	Ha 63**	7.43	*W. anomalus*	P01A017	0.36

Strains obtained from a regional winery (*), ARS-NRRL culture collection (**), or Lallemand Inc., Montréal, Quebec, Canada (***).

Table 12. Polygalacturonase activity of various yeasts and compositional analysis after grown in grape juice.

Species/Strain	Uronic Acid (μmol/mL GALA eq.)	pH	Titratable Acidity (g/L)	Volatile Acidity (g/L)
Cr. adelienses P44A007	6.08	3.69	7.11	0.41
Cr. macerans P41D001	4.14	3.66	7.20	0.30
I. orientalis J5-6-2*	5.60	3.53	7.41	0.36
K. marxianus Ha 63**	5.03	3.52	7.32	0.38
P. kluyveri P01C002	5.57	3.56	7.41	0.40
S. cerevisiae ICV D254***	5.90	3.49	7.87	0.45

Strains obtained from a regional winery (*), ARS-NRRL culture collection (**), or Lallemand Inc., Montréal, Quebec, Canada (***).

Information Dissemination, Extension, and Outreach Activities (FY 2015-2018):

Publications (graduate students indicated by "*" and presenter by "†")

Zuehlke*, J.M., D.A. Glawe, and C.G. Edwards. Efficacy of dimethyl dicarbonate against yeasts associated with Washington State grapes and wines. *J. Food Proc. Pres.* 39: 1016-1026 (2015).

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Zuehlke*, J.M., B.C. Childs*, and C.G. Edwards. Evaluation of *Zygosaccharomyces bailii* to metabolize residual sugar present in partially-fermented red wines. *Fermentation* 1: 3-12 (2015).

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Strickland*, M.T., L.M. Schopp*, C.G. Edwards, and J.P. Osborne. Impact of *Pediococcus* spp. on Pinot noir wine quality and growth of *Brettanomyces*. *Am. J. Enol. Vitic.* 67: 188-198 (2016).

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Wade*, M.E., J.P. Osborne, S.C. Chescheir*, and C.G. Edwards. Role of *Pediococcus* spp. in winemaking. *Aust. J. Grape Wine Res.* (in revision, 2018).

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Aplin*, J.A., K.P. White*, and C.G. Edwards. Enological characterization of non-*Saccharomyces* yeasts strains isolated from Washington state vineyards. *Food Microbiol.* (in preparation, 2018).

Aplin*, J.A., and C.G. Edwards. Ethanol reduction using sequential inoculations of non-*Saccharomyces* yeasts. *Aust. J. Wine Grape Res.* (in preparation, 2018).

Cartwright*, Z.M. and C.G. Edwards. Survival of *Brettanomyces bruxellensis* populations in grape pomace. *Am. J. Enol. Vitic.* (in preparation, 2018).

Abstracts and Papers Presented at Technical Meetings

Aplin†*, J. and C.G. Edwards. Characterization of non-*Saccharomyces* yeasts found In Washington State vineyards. Washington Association of Wine Grape Growers annual meeting, Kennewick, WA. February 10-13 (2015).

Wang†*, X., C.G. Edwards, and D.A. Glawe. Persistence of indigenous grape yeast species during alcoholic fermentation. Washington Association of Wine Grape Growers annual meeting, Kennewick, WA. February 10-13 (2015).

Cartwright†*, Z.M. and C.G. Edwards. Evaluation of the survival of *Brettanomyces bruxellensis* in grape pomace. Washington Association of Wine Grape Growers annual meeting, Kennewick, WA. February 10-13 (2015).

Diako*, C., Z. Cartwright†*, C.G. Edwards, and C.F. Ross. Application of the electronic tongue in the evaluation of wine faults. Washington Association of Wine Grape Growers annual meeting, Kennewick, WA. February 10-13 (2015).

Cartwright†*, Z.M. and C.G. Edwards. Evaluation of the survival of *Brettanomyces bruxellensis* in grape pomace. Dr. William R. Wiley Research Exposition, Washington State University, Pullman, WA. February 20 (2015).

Cartwright†*, Z.M. and C.G. Edwards. Examining *Brettanomyces bruxellensis* survival in grape pomace over time in Washington State vineyards. Annual meeting of the Institute of Food Technologists, Chicago, IL. July 11-14 (2015).

Diako†*, C., K. McMahon*, M.A. Evans, C.G. Edwards, and C.F. Ross. Interactions among alcohol, tannins, and mannoproteins influence the sensory properties of commercial Merlot wines. Presented at the annual meeting of the Institute of Food Technologists, Chicago, IL. July 11-14 (2015).

Aplin, J.J.†*, D.A. Glawe, N.L. Rivera, and C.G. Edwards. Screening of native non-*Saccharomyces* yeast isolates for reducing ethanol production. Washington Association of Wine Grape Growers annual meeting, Kennewick, WA. February 8-10 (2016).

Cartwright†*, Z.M., D.A. Glawe, and C.G. Edwards. Analysis of *Brettanomyces bruxellensis* in different types of oak staves. Washington Association of Wine Grape Growers annual meeting, Kennewick, WA. February 8-10 (2016).

Hogrefe-O-Regan, N.†* and C.G. Edwards. Use of fining techniques and alternative processing strategies to limit *Brettanomyces bruxellensis* infections in red wines. Washington

Association of Wine Grape Growers annual meeting, Kennewick, WA. February 8-10 (2016).

Henick-Kling, T., H. Piao, P. Okubara, C. Edwards, T. Murray, D. Glawe, and M. Hess. A look into the microbial populations of Washington State vineyards and their persistence during wine fermentation. Presented at XXVIth Entretiens Scientifiques Lallemand on Biodiversity Meets Terroir. Osoyoos, British Columbia, Canada. April 28 (2016).

Aplin IV†*, J.J., D.A. Glawe, N.L. Rivera, and C.G. Edwards. Evaluation of native non-*Saccharomyces* yeasts for reducing ethanol production in wine by sugar respiration. Presented at American Society for Enology and Viticulture annual meeting, Monterey, CA. June 27-30 (2016).

Cartwright†*, Z.M. D.A. Glawe, and C.G. Edwards. Analysis of *Brettanomyces bruxellensis* penetration depths in different types of oak barrel staves. Presented at American Society for Enology and Viticulture annual meeting, Monterey, CA. June 27-30 (2016).

Cartwright†*, Z.M. D.A. Glawe, and C.G. Edwards. Analysis of the wine spoilage microorganism *Brettanomyces bruxellensis* in different types of oak barrels. Presented at Institute of Food Technologists annual meeting, Chicago, IL. July 16-19 (2016).

Cartwright†*, Z.M., D.A. Glawe, and C.G. Edwards. Eradication of the spoilage yeast, *Brettanomyces bruxellensis*, from oak barrels using various heat treatments. Institute of Food Technologists annual meeting, Las Vegas, NV, June 25-28 (2017).

Cartwright†*, Z.M. and C.G. Edwards. Survival of *Brettanomyces bruxellensis* in grape pomace. American Society for Enology and Viticulture annual meeting, Bellevue, WA, June 26-29 (2017).

Cartwright†*, Z.M. and C.G. Edwards. Reduction of *Brettanomyces bruxellensis* populations from oak barrel staves using steam. American Society for Enology and Viticulture annual meeting, Bellevue, WA, June 26-29 (2017).

Wade*†, M.E., J.P. Osborne, and C.G. Edwards. Impact of nutrient supplementation of synthetic grape juice on growth of *Pediococcus* spp. post-alcoholic fermentation. American Society for Enology and Viticulture annual meeting, Bellevue, WA, June 26-29 (2017).

Aplin*†, J.J. and C.G. Edwards. Sequential inoculation of different yeasts to reduce alcohol contents of red wines. American Society for Enology and Viticulture annual meeting, Bellevue, WA, June 26-29 (2017).

Oswald*, T.A. and C.G. Edwards†. Impact of storage temperature and ethanol on *Brettanomyces bruxellensis* inoculated into Merlot wine. American Society for Enology and Viticulture annual meeting, Bellevue, WA, June 26-29 (2017).

Okubara†, P., X. Wang*, D. Schlatter, C. Edwards, T. Paulitz, and D. Glawe. Native yeast diversity in two Washington Cabernet Sauvignon vineyards consists of common and unique

species. American Society for Enology and Viticulture annual meeting, Bellevue, WA, June 26-29 (2017).

Invited Presentations

Edwards†, C.G. Management of *Brettanomyces*. Presented at the Ste. Michelle Wine Estates Winemaker Council meeting, Clore Wine Center, Prosser, WA, May 27 (2015).

Edwards†, C.G. Nutritional needs of *Saccharomyces* and *Brettanomyces*. Presented at the British Columbia Wine Grape Council annual enology & viticulture conference. Penticton, BC, Canada, July 20-21 (2015).

Edwards†, C.G. Wine Microbes and Control. Presented at the Idaho Wine Commission annual meeting, Boise, ID, February 24 (2016).

Edwards†, C.G. New methods towards managing *Brettanomyces*. Presented at the Washington Technical Group, Richland, WA, March 23 (2016).

Edwards†, C.G. What we've learned about wine spoilage and *Brettanomyces*. Presented at Washington Advancements in Viticulture and Enology meeting, Richland, WA July 14 (2016).

Edwards†, C.G. Wine fermentation: why does my red wine smell odd? Presented at the Washington State University Association of Faculty Women, Pullman, WA May 6 (2016).

Edwards†, C.G. Wine fermentation: why does my red wine smell odd? Presented at the University Creamery Managers Association, Pullman, WA June 14 (2016).

Edwards†, C.G. Wine fermentations: Why does my red wine smell odd? Presented as part of the "Science After Hours" series hosted by Palouse-Clearwater Environmental Institute, Moscow, ID October 6 (2016).

Edwards†, C.G. What we've learned about wine spoilage and *Brettanomyces*. Oregon Wine Symposium, Portland, OR February 22 (2017).

Edwards†, C.G. What we've learned about wine spoilage and *Brettanomyces*. E&J Gallo Technical Seminar, Modesto, CA May 12 (2017).

Edwards†, C.G. What we've learned about wine spoilage and *Brettanomyces*. Presented at the Ste. Michelle Wine Estates Winemaker Council meeting, Clore Wine Center, Prosser, WA, June 6 (2017).

Edwards†, C.G. The Bisson retirement: What have we really witnessed over these years? Presented at Wine Flavor 101A Managing Wine Quality: Problematic Fermentation symposium, University of California (Davis), Davis, CA, February 15 (2018).

Edwards†, C.G. Managing brett in the winery. Presented at the Washington Advancements in Viticulture and Enology annual meeting, Clore Wine Center, Prosser, WA, April 4 (2018).